The EMBO Journal vol.12 no.4 pp.1671 – 1680, 1993

A dominant-negative transgene defines a role for p56^{lck} in thymopoiesis

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Communicated by H.Eisen

The lymphocyte-specific protein tyrosine kinase p56^{lck} participates in T cell signaling through functional interactions with components of the T cell antigen receptor complex and the interleukin-2 receptor. Additional insight into the function of p56^{lck} has now been obtained through the generation of transgenic animals expressing high levels of a catalytically inactive form of this kinase (p56^{lckR273}). Mice bearing the *lck*R273 transgene manifested a severe defect in the production of virtually all T lymphocytes. Those exceptional CD3$^+$ cells that escaped the effects of the *lck*R273 transgene were confined primarily to the T cell subset that expresses γ/δ T cell receptors. Remarkably, construction of a dose–response curve for the effects of the *lck*R273 transgene revealed that developmental arrest of thymocytes occurred at a discrete stage in the normal T cell maturation pathway, corresponding to a point at which thymoblasts ordinarily begin a series of mitotic divisions that result in expansion and maturation. These results suggest that p56^{lck} normally regulates T cell production by metering the replicative potential of immature thymoblasts.

Key words: p56^{lck}/T cells/thymopoiesis

Introduction

The *lck* gene was first identified by virtue of its overexpression in certain rare retrovirally-induced murine T cell malignancies (Marth *et al.*, 1985; Voronova and Sefton, 1986; Adler *et al.*, 1988) and encodes a lymphocyte-specific membrane-associated protein tyrosine kinase (p56^{lck}) of the non-receptor type (Marth *et al.*, 1985; Perlmutter *et al.*, 1988; Cooper, 1989). Overexpression of p56^{lck} promotes tumorigenesis in otherwise normal murine thymocytes (Abraham *et al.*, 1991a,c). In addition, the human *lck* gene, which encodes a nearly identical protein, is positioned at a site of frequent chromosomal abnormalities in human lymphoid cancers (Marth *et al.*, 1986).

These data suggesting a role for p56^{lck} in the control of T cell growth complement other studies documenting the involvement of p56^{lck} in signaling pathways that regulate lymphocyte activation. For example, p56^{lck} associates physically and functionally with the CD4 and CD8 coreceptors that assist in antigen recognition by T cells (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Barber *et al.*, 1989). Alterations in CD4 or CD8 that affect interaction with p56^{lck} compromise coreceptor-mediated signaling in both mature T cells (Zamoyska *et al.*, 1989; Letourneur *et al.*, 1990; Chalupny *et al.*, 1991; Glaichenhaus *et al.*, 1991) and developing thymocytes (van Oers *et al.*, 1992). Moreover, in some cases overexpression of a catalytically activated version of p56^{lck} can improve the response of T cell lines challenged with appropriate antigens (N.Abraham *et al.*, 1991). Finally, the activity of p56^{lck} in normal circulating T cells increases dramatically following treatment with the potent T cell growth factor interleukin-2 (IL-2) and p56^{lck} can be found physically associated with the β chain of the IL-2 receptor (Hatakeyama *et al.*, 1991; Horak *et al.*, 1991). Hence p56^{lck} can relay signals from at least three different receptors in mature T lymphocytes.

Examination of the contribution of p56^{lck} to signaling in mature T cells has to some extent diverted attention from the fact that *lck* transcripts accumulate to very high levels in developing thymocytes (Marth *et al.*, 1985; Perlmutter *et al.*, 1988; Bolen *et al.*, 1991; Wildin *et al.*, 1991; Reynolds *et al.*, 1990), though few of these cells express either antigen or IL-2 receptors (Palacios and Pelkonen, 1988; Scollay *et al.*, 1988; von Boehmer, 1988). While no satisfactory *in vitro* models of thymocyte development exist, identification of the transcriptional regulatory elements that direct thymocyte-specific expression of the *lck* gene (Garvin *et al.*, 1988; Wildin *et al.*, 1991; Allen *et al.*, 1992) has permitted us to perform an *in vivo* dissection of the function of p56^{lck} during T cell development (Abraham *et al.*, 1991a,b,c). We have now extended this analysis using a dominant-negative approach. Our strategy evolved from recent studies of receptor-type protein tyrosine kinases, particularly the naturally occurring mutants of the c-*kit* kinase (Nocka *et al.*, 1989; Tan *et al.*, 1990), where catalytically inactive forms of the receptor behave in a dominant-negative fashion. In addition, overexpression of a catalytically inactive form of the p59^{fyn} non-receptor protein tyrosine kinase in mature thymocytes cleanly disrupts the signaling behavior of these cells (Cooke *et al.*, 1991). We now report that a catalytically inactive version of p56^{lck} also behaves in a dominant negative fashion, but impinges on a process quite distinct from that regulated by p59^{fyn}. Aside from revealing the remarkable specificity of these two closely-related protein tyrosine kinases, our experiments permit definition of a point during thymocyte development when p56^{lck} activity is critical for all subsequent maturational events.

Results

Design of transgenic expression constructs

Since p56^{lck} is ordinarily expressed at high levels in thymocytes, assembly of a transgene that could direct expression of competitive levels of a catalytically inactive

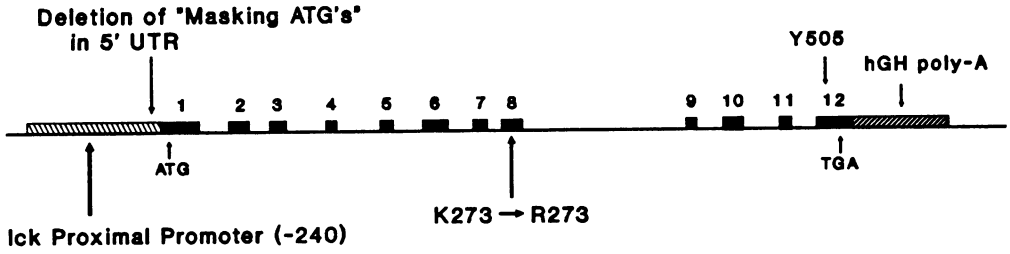

Fig. 1. Transgene expression vector. The *lck*R273 construct contains 9.6 kb of the *lck* gene (exons 1–12) with the lysine codon at position 273 changed to an arginine codon. This sequence was fused to 240 bp of *lck* proximal promoter (at the 5′ end) and to the 3′ untranslated region from the human growth hormone gene (hGH) which was positioned after the termination codon in the *lck* coding sequence. Three 'masking ATGs' in the 5′ untranslated region were deleted to enhance translational efficiency.

version of $p56^{lck}$ proved arduous. Design of an effective construct benefited from detailed studies of the *lck* proximal promoter, which controls the majority of thymocyte-specific *lck* transcription (Reynolds *et al.*, 1990; Wildin *et al.*, 1991; Allen *et al.*, 1992). The final 13 kb construct included all 12 mouse *lck* coding exons and a 240 bp promoter fragment previously shown to direct high level transgene expression in a tissue-specific and temporally-correct fashion (Allen *et al.*, 1992). To maximize translation of the transgene-encoded mRNA, a 125 bp region of exon 1, encoding 5′ untranslated region sequences previously shown to reduce translational efficiency (Marth *et al.*, 1988; Perlmutter, 1990) was excised. Site-directed mutagenesis was used to replace the lysine codon corresponding to residue 273 with an arginine codon. Previous studies had demonstrated that the analogous lysine residue in the $p59^{fyn}$ and $p59^{hck}$ protein tyrosine kinases was required for satisfactory phosphate transfer (Ziegler *et al.*, 1989; Cooke *et al.*, 1991). Finally, a 3′ untranslated region sequence derived from the human growth hormone gene was added to provide a marker for transgene integration and expression (p*lck*R273, Figure 1). Transgenic animals were generated by injection of C57BL/6 X DBA/2 F_2 mouse embryos and 11 independent lines of the resulting *lck*R273 transgenic mice were analyzed. In each line, expression of the transgene was limited to the thymus (data not shown), as had been previously noted using similar constructs (Cooke *et al.*, 1991; Abraham *et al.*, 1991b,c; Allen *et al.*, 1992). Figure 2 demonstrates that thymocytes from transgenic animals contained increased levels of immunoreactive $p56^{lck}$ protein, which would be expected to differ by only a single amino acid from the endogenous kinase and hence could not be distinguished from wild-type product by electrophoretic properties. However, the specific activity of $p56^{lck}$ in immunoprecipitates from these extracts was substantially decreased, reflecting the accumulation of catalytically inactive protein (data not shown).

Thymocyte development requires catalytically active $p56^{lck}$

The availability of mice that expressed various levels of the *lck*R273 transgene product permitted the establishment of a dose–response curve for its effects. Transgenic animals exhibited a dramatic decrease in thymic size, which correlated well with the level of transgene expression. Figure 3 documents this dose–response relationship for 10 different lines of *lck*R237 mice. At transgene expression levels corresponding to an ~12-fold increase in total $p56^{lck}$ protein, thymocyte number was reduced to <2% of the number of thymocytes found in littermate control mice. Not

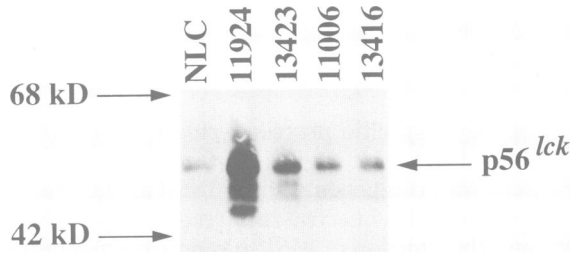

Fig. 2. $p56^{lck}$ expression in *lck*R273 transgenic mice. Whole-cell lysates of 5×10^5 thymocytes (~ 4 μg total protein) from *lck*R273 transgenic mice or control animals (NLC) were resolved on 10% SDS–PAGE and transferred to nitrocellulose. The filter was then blotted with an anti-$p56^{lck}$ antiserum (195.7) as described in Materials and methods. The positions of molecular weight standards and $p56^{lck}$ are indicated with arrows.

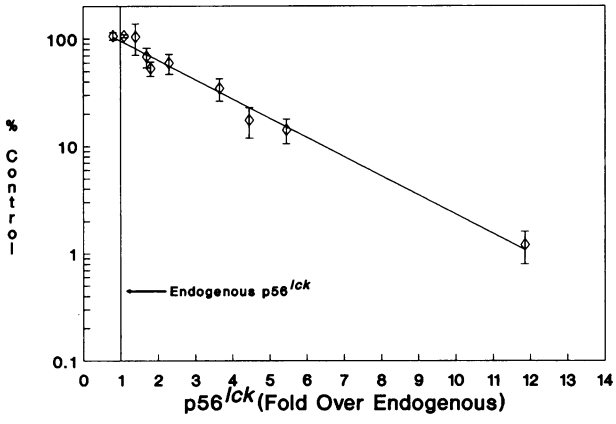

Fig. 3. Effect of transgene expression on thymocyte number in *lck*R273 transgenic mice. Total thymocyte number was determined for ten lines of mice transgenic for the *lck*R273 construct and compared to age-matched normal littermate controls. Thymocyte numbers are expressed as mean percent of control values ± SEM (or in one case range) and were derived from 2–6 transgene-positive mice per line (see Table I). Control values were determined from at least two animals per experiment. Thymocyte numbers are plotted on a log scale against total $p56^{lck}$ determined simultaneously from immunoblots of thymocyte extracts (see Table I).

surprisingly, peripheral T lymphocyte counts were also dramatically reduced in these animals (Table I), although the representation of normal appearing $CD4^+$ and $CD8^+$ cells was compromised to a lesser extent in secondary lymphoid organs, presumably reflecting the peripheral expansion of mature T lymphocytes in which the *lck* proximal promoter, and hence the *lck*R273 gene, is transcrip-

Table I. Summary of parameters affected by the *lck*R273 transgene

Line	*n*	p56^{lck} level	Thymus cell number	4$^-$/8$^-$ cell number	4$^+$/8$^+$ cell number	Peripheral T cells	
						Splenic	Inguinal LN
11924	8	11.8 ± 1	1.2 ± 0.4	40 ± 23	0.03 ± 0.01	16 ± 3	9 ± 2
10265	1	ND	8	50	6	37	49
13413	4	5.4 ± 1.2	14 ± 4	98 ± 23	10 ± 4	68 ± 8	76 ± 6
11711	4	4.4 ± 0.4	17 ± 6	78 ± 15	12 ± 4	62 ± 6	78 ± 2
13423	7	3.6 ± 0.5	34 ± 8	84 ± 8	29 ± 8	78 ± 8	88 ± 4
13416	3	2.3 ± 0.3	59 ± 12	92 ± 3	56 ± 14	94 ± 13	98 ± 0.3
11006	5	1.8 ± 0.2	53 ± 8	61 ± 8	51 ± 9	97 ± 12	100 ± 5
11927	6	1.7 ± 0.1	74 ± 11	81 ± 12	73 ± 12	82 ± 8	102 ± 3
13390	2	1.4 ± 0.6	104 ± 33	100 ± 37	104 ± 34	99 ± 7	99 ± 5
10271	6	1.1 ± 0.1	106 ± 3	98 ± 11	107 ± 2	96 ± 5	94 ± 4
10259	6	0.8 ± 0.2	106 ± 8	98 ± 12	108 ± 9	97 ± 9	102 ± 4
*lck^{null}	NR	≤ 0.05	3–10	50–100	2–9	NR	7

Values in each case are percentage of littermate control values for the indicated parameter, with the exception of p56^{lck} levels which are presented as fold over endogenous. Peripheral T cells were characterized by staining with antibody to Thy1.2. NR = not reported. ND = not determined.

tionally inactive (Wildin *et al.*, 1991). Mice in which transgene expression was less robust exhibited more modest defects in the accumulation of T lineage cells.

Insight into the nature of this disturbance was obtained by flow cytometric examination of the *lck*R273 thymocytes using markers that define specific stages in thymocyte development. In normal mice, immature hematopoietic progenitors that colonize the thymus initially fail to express either the T cell antigen receptor (TCR) and its associated CD3 complex or the coreceptor components CD4 or CD8 (Palacios and Pelkonen, 1988; Scollay *et al.*, 1988; von Boehmer, 1988). These cells localize within the subcapsular region of the thymus and, together with a slightly more mature subset that bears low levels of the CD8 molecule, undergo several rounds of replication to yield CD4$^+$8$^+$ thymocytes that constitute >80% of thymic cells in young mice. CD4$^+$8$^+$ cells successively acquire low level expression of the TCR–CD3 complex (often revealed using antibodies to the CD3ε component), undergo selection that eliminates self-reactive cells and retains those with functional antigen receptors (negative and positive selection, respectively) and differentiate into cells that express high levels of the TCR–CD3 complex and CD4 or CD8 in a mutually exclusive fashion. In *lck*R273 mice, a dose-dependent reduction in all thymic subsets except those that lack CD4 and CD8 expression (the most immature cells) was observed. Figure 4 shows representative two-parameter CD4 compared with CD8 flow cytometric histograms for four independent lines of *lck*R273 mice documenting the relative preservation of the CD4$^-$8$^-$ population which in normal animals constitutes <5% of total thymocytes. The absolute numbers of such cells were not dramatically diminished even in the most severely affected *lck*R273 animals (the 11924 line; see Table I). However, in these animals all other thymocyte subpopulations were essentially eliminated.

Arrested thymocyte maturation spares γ/δ bearing cells

Although most CD4$^-$8$^-$ thymocytes in normal animals fail to express the TCR–CD3 complex, as many as 20% of thymocytes in severely affected *lck*R273 mice were CD3$^+$. Remarkably, these cells failed to stain using the H57 anti-TCRβ antibody, but clearly expressed TCRδ chains (Figure 5). Previous studies support the view that γ/δ cells develop along a pathway distinct from that which yields the bulk of T lymphocytes bearing α/β T cell receptors (Winoto and Baltimore, 1989; Dent *et al.*, 1990). Although the details of this pathway remain to be elucidated, our experiments suggest that p56^{lck} function is not required to permit production of γ/δ bearing cells in the thymus. An alternative interpretation, that the *lck* promoter driving the transgene is inactive in γ/δ precursors, seems much less likely; we have previously shown that the *lck* proximal promoter element directs expression of heterologous genes in all thymocytes including those bearing γ/δ receptors (Teh *et al.*, 1991; data not shown). Hence, the maturation process that permits development of mature CD4$^+$ and CD8$^+$ T cells that bear the α/β receptor was fatally compromised by overexpression of catalytically inactive p56^{lck}.

Defective thymocyte mitogenesis in lckR273 mice

Figure 6 presents a schematic view of known thymocyte maturation events defined by other groups. This scheme was formulated primarily through multiparameter analysis of thymocytes developing in lethally irradiated mice reconstituted with hematopoietic progenitors (Scollay *et al.*, 1988; Boyer *et al.*, 1989; Egerton *et al.*, 1990). Although all early T cell precursors lack CD3, CD4 and CD8 expression, they differ with respect to size and also vary with respect to the expression of other cell surface structures, notably CD44 (Pgp1), the heat-stable antigen (HSA) and the IL-2 receptor α chain (reviewed in Scollay *et al.*, 1988). Remarkably, thymocytes from severely affected lines of *lck*R273 animals manifested a nearly homogeneous appearance. Most of these cells had the size characteristics of thymoblasts (they are 20–25% larger than the bulk of normal resting thymocytes) and were predominantly HSA$^+$ CD44$^-$IL-2Rα$^+$ (Figure 7 and data not shown). Hence with reference to the maturation scheme shown in Figure 6, the *lck*R273 thymocytes appeared to be arrested at a characteristic stage defined by size and cell surface protein expression.

In principle, the reduction in thymocyte numbers observed in *lck*R273 animals could result from decreased production of thymocytes or from increased destruction of these cells as they mature. Several lines of evidence favor the former

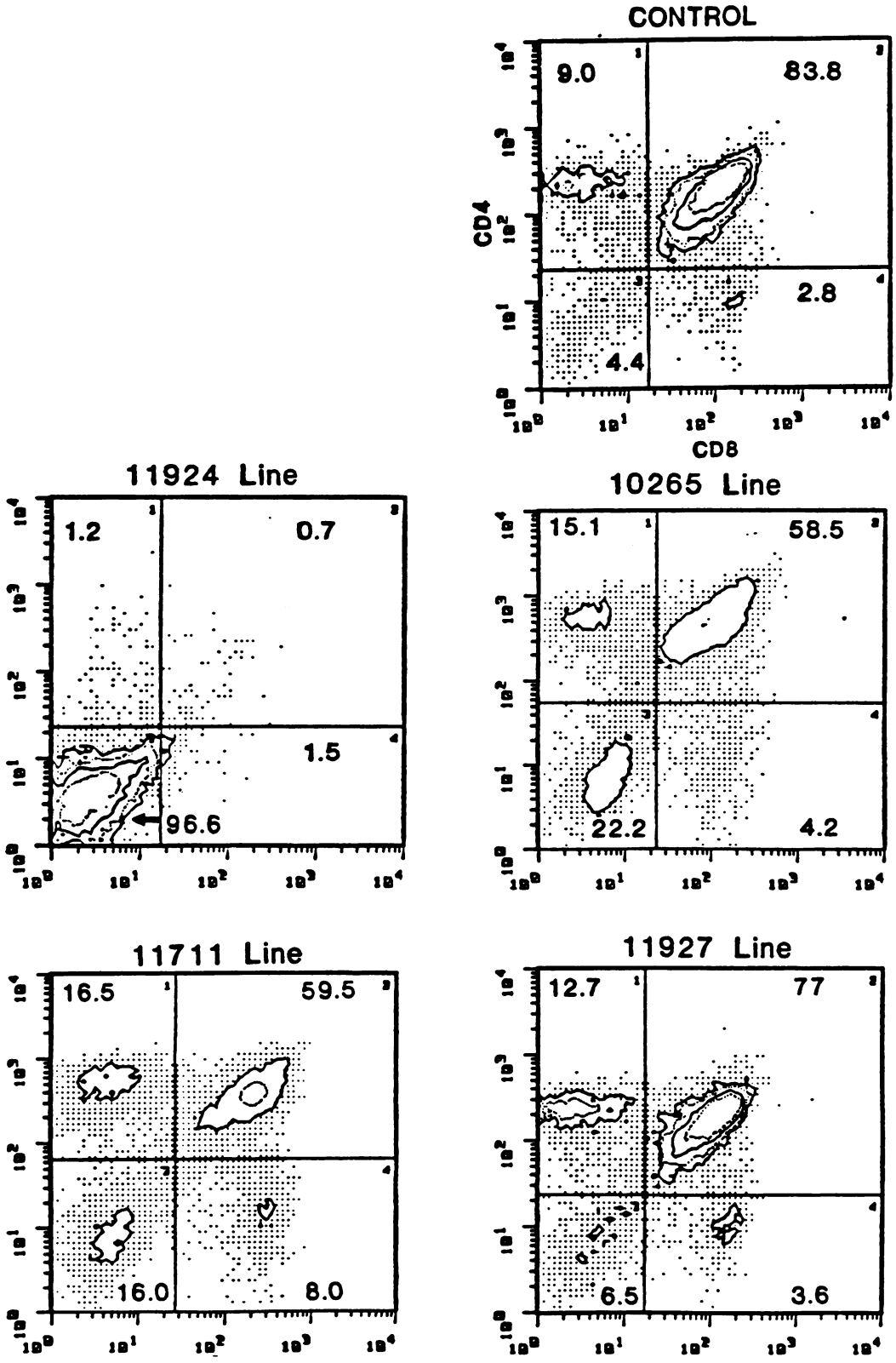

Fig. 4. Thymocyte CD4 × CD8 profiles from *lck*R273 mice. Thymocytes from representative mice were stained for CD4 and CD8 expression (see Materials and methods). Shown are two-dimensional flow cytometric histograms for four transgenic lines compared to a typical control animal. The percentages of cells falling into each quadrant are indicated.

interpretation. For example, as shown in Figure 3 and Table I, increased expression of $p56^{lckR273}$ was linearly related to the logarithm of thymocyte number. This relationship held for animals with thymuses ranging from 10–90% of normal size and appeared to extrapolate to the most severely affected animals. Since thymopoiesis is an exponential process and mitotic expansion in the thymus ordinarily begins in $CD4^-8^-$ cells with characteristics like those observed in

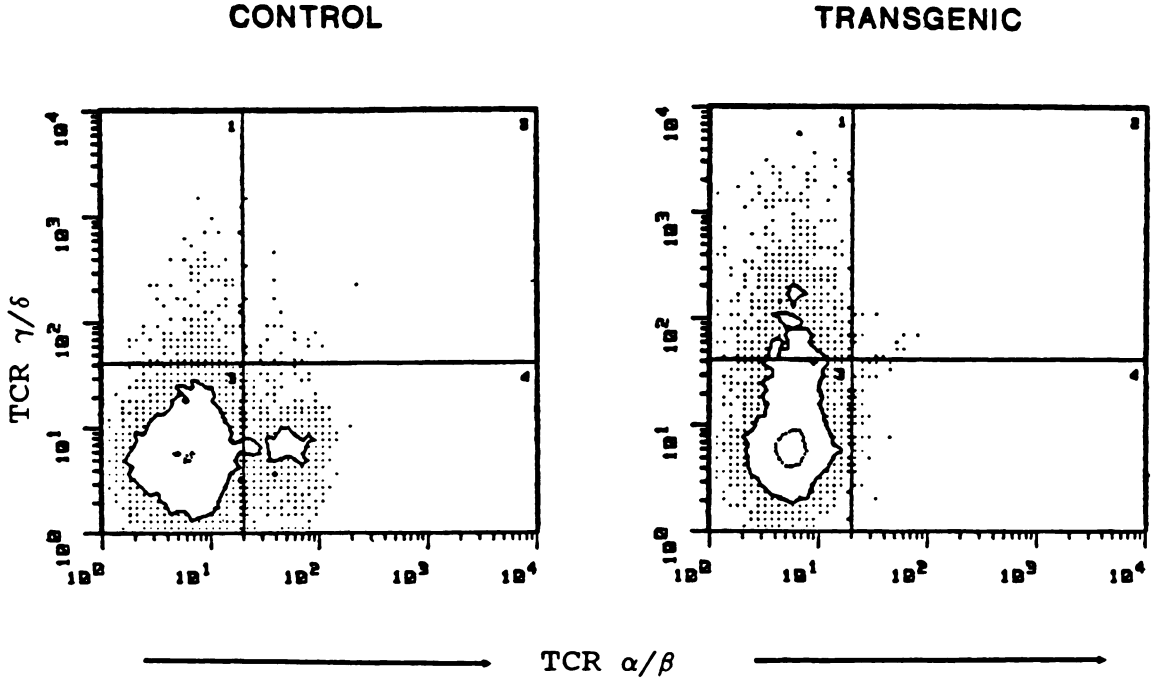

Fig 5. Cells bearing α/β versus γ/δ TCRs in *lck*R273 11924 thymocytes. Thymocytes from a control and an *lck*R273 animal were stained for γ/δ TCR versus α/β TCR as described in Materials and methods. Notably, none of the cells in the transgenic thymus stained brightly with the Cβ reagent, although a significant fraction did stain using anti-TCRδ.

*lck*R273 mice, it seemed possible that *lck*R273 expression directly interfered with thymocyte replication.

To evaluate this hypothesis, we examined the extent to which *lck*R273 thymocytes transit the cell cycle. Table II presents the results of DNA content determinations performed in normal thymocytes, in normal CD4$^-$8$^-$ thymocytes and in thymocytes derived from representative *lck*R273 mice of the severely affected 11924 line. Between 8 and 9% of normal thymocytes exhibited $>2N$ DNA content, reflecting the low level of mitogenesis usually observed (Egerton *et al.*, 1990) and the fact that the cell cycle in dividing thymocytes consumes only ~10 h (Baron and Penit, 1990). The representation of cycling cells was much enriched when gating on immature CD4$^-$8$^-$ cells (Table II), as previously reported by others (Scollay *et al.*, 1988; Penit *et al.*, 1988; Egerton *et al.*, 1990). In contrast, CD4$^-$8$^-$ cells in *lck*R273 mice remained almost entirely confined to the G_0/G_1 phase of the cell cycle, demonstrating at least an 80% reduction in the proportion of cells with $>2N$ DNA content as compared with similar CD4$^-$8$^-$ cells derived from littermate control thymuses. We conclude that thymocyte-specific overexpression of a catalytically inactive form of p56^{lck} severely disturbed T cell development, resulting in a quite specific maturational arrest that preserved G_0/G_1 thymoblasts, but failed to permit replication.

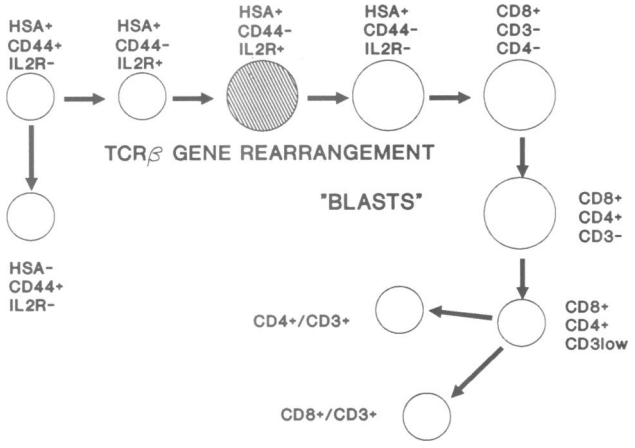

Fig. 6. Schematic diagram of early thymocyte development. Cell populations with characteristic phenotypes are indicated by circles, with arrows illustrating proposed precursor–product relationships (Scollay *et al.*, 1988; Boyer *et al.*, 1989; Egerton *et al.*, 1990). The larger circles denote cells with 'blast' characteristics; cell surface staining properties are also indicated. The cell type which makes up the bulk of thymocytes in the *lck*R273 11924 line is shaded. See text for details.

T cell receptor gene rearrangements in lckR273 mice

Expression of the TCR–CD3 complex requires gene rearrangement events that proceed in a developmentally ordered fashion, such that β chain gene rearrangements occur prior to α chain rearrangements (Snodgrass *et al.*, 1985a,b). Since T cell receptor gene rearrangements begin in immature CD3$^-$4$^-$8$^-$ thymocytes (see Figure 6), we examined the status of the T cell receptor genes in thymocytes from severely affected 11924 *lck*R273 thymocytes using a PCR-based method (van Meerwijk *et al.*, 1990; Anderson *et al.*, 1992). Vβ–Dβ–Jβ joining events were detectable at equivalent levels in wild-type CD4$^-$8$^-$ cells and *lck*R273 thymocytes. Rearrangements of Vβ12 are shown in Figure 8 and similar results were obtained using primers specific for Vβ4, Vβ5, Vβ8, and Vβ11 (data not shown). However, whereas products of Vα–Jα joining events were detected in normal CD4$^-$8$^-$ thymocytes, no such products appeared in the *lck*R273 cells (Figure 8; data not shown). The fact that Vβ–Dβ rearrangements are detected indicates the presence of α/β lineage precursors, since γ/δ cells generally

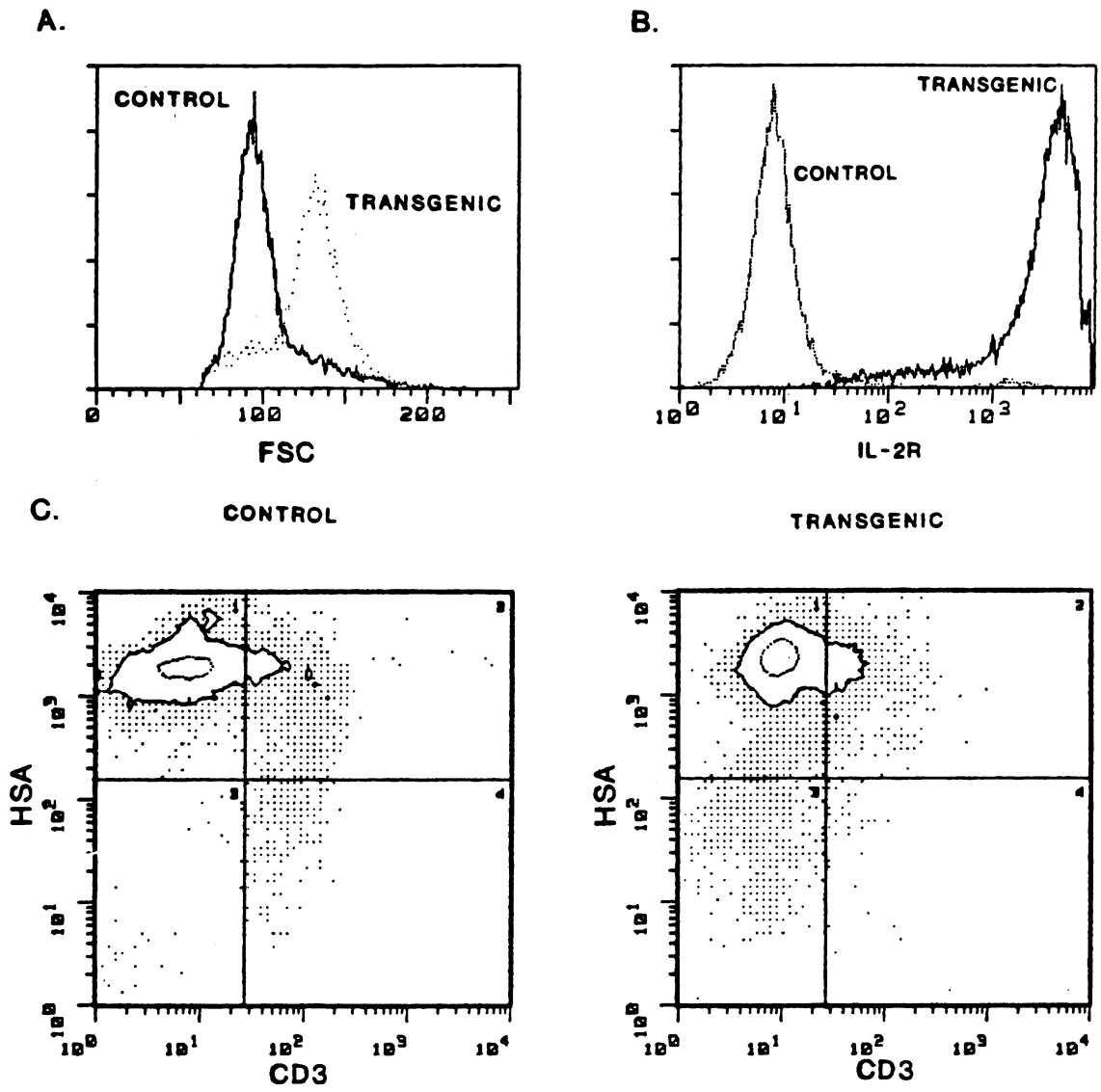

Fig. 7. Cell surface phenotype of *lck*R273 11924 thymocytes. (**A**) Shown are the forward light scatter (FSC) profiles (plotted versus cell number on the ordinate) for thymocytes from 11924 *lck*R273 transgenic mice (dotted line) as compared with those obtained from a littermate control animal (solid line). (**B**) The expression of IL-2 receptor α chain on 11924 transgenic thymocytes was assessed using single-parameter flow cytometry (plotted versus cell number on the ordinate). Compared with littermate control thymocytes, virtually all of the transgenic cells express high levels of this protein. (**C**) Shown are two-parameter flow cytometric histograms of heat stable antigen (HSA) versus CD3 expression for thymocytes from a representative 11924 *lck*R273 transgenic animal and a littermate control. $CD3^+HSA^-$ cells (representing the most mature thymocytes) are largely absent from profiles of transgenic mice, which instead contain more $CD3^-HSA^-$ cells.

do not support Vβ–Dβ joining (Marusic-Galesic *et al.*, 1988; Raulet *et al.*, 1991). Hence the maturational arrest induced by overexpression of $p56^{lckR273}$ produced G_0/G_1 thymoblasts of the α/β lineage that have achieved β chain, but not α chain, gene rearrangements.

Discussion

$p56^{lck}$ regulates thymocyte maturation

Overexpression of a catalytically inactive version of $p56^{lck}$ severely compromised normal thymocyte maturation. In animals expressing high levels of the inactive kinase, thymocyte number was reduced >80-fold and most of those cells remaining were confined to a single somewhat unusual subset: $CD3^-4^-8^-$ lymphoblasts bearing rearranged β chain genes but not α chain genes. Several observations suggest that these results reflect inhibition of a $p56^{lck}$ regulated signaling pathway. In particular, the catalytically inactive $p56^{lckR273}$ protein interfered with thymocyte maturation in a dose-dependent way and in the most severe cases yielded cells similar to those observed in the thymuses of mice bearing an lck^{null} mutation (Molina *et al.*, 1992 and

Table II. Percent of thymocytes with DNA content >2*N*

Animal	Total thymocytes	$CD4^-/CD8^-$ cells
NLC	8.9	19.7
NLC	8.6	19.3
11924*lck*R273	3.5	3.5
11924*lck*R273	2.8	2.8

DNA content determinations were done (see Materials and methods) on thymocytes from two separate 11924*lck*R273 animals and two littermate controls (NLC).

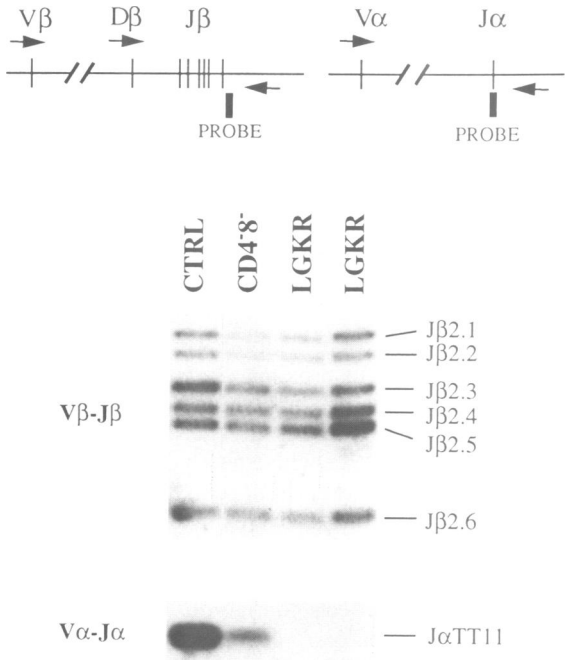

Fig. 8. Detection of Vβ, but not Vα, rearrangements in *lck*R273 thymocytes. Schematic representation of PCR primers (arrows) and probes (bars) is shown. PCR products specific for Vβ12 with Jβ2 (top) or a mixture of VαF3, Vα2c and VαH with JαTT11 (bottom) were visualized after blot hybridization with the probes indicated at top. Ctrl, total normal thymocytes; $CD4^-8^-$, thymocytes isolated from a control animal after lysis with anti-CD4 and CD8 plus complement; LGKR, thymocytes (virtually all $CD4^-8^-$) from two separate animals of the *lck*R273 11924 line. The positions of specific rearrangements are marked.

see below). What then can be said of the function of $p56^{lck}$ in thymocyte development?

$p56^{lck}$ activity regulates thymocyte mitogenesis

Measurement of cellular DNA content established that the representation of $CD4^-8^-$ cells in $S/G_2/M$ phases of the cell cycle in severely affected *lck*R273 mice declined from >19% to <4%. This suggested that thymocyte production, rather than thymocyte survival, was most severely altered by expression of the transgene. It is worth noting that the absolute number of immature $CD4^-8^-$ cells in the *lck*R273 mice was in all cases relatively unaffected (Table I). Together these results argue that generation of $CD4^-8^-$ thymoblasts occurs via a $p56^{lck}$-independent mechanism. In contrast, significant expansion of this precursor population appears to require active $p56^{lck}$ protein. Indeed, a previous report demonstrated that mice lacking $p56^{lck}$ expression, like the most severely affected *lck*R273 animals, retained a very small number of thymocytes, nearly 50% of which were $CD4^-8^-$ cells (Molina *et al.*, 1992; and see below). If the dose–response presented in Figure 3 fairly represents the effects of $p56^{lckR273}$, the irreducible number of thymocytes that can exist in an lck^{null} mouse probably represents ~1% of that found in normal animals (Figure 3). Under these circumstances, a minimum of 6–7 cell divisions ($2^7 = 128$) must separate these immature progenitors from the final products of thymopoiesis, provided that all divisions occur symmetrically. Since in adoptive transfer experiments even a few thymocyte progenitors can give rise to large numbers of mature thymocytes (Scollay *et al.*, 1988), it appears that all mitotic divisions separating $CD4^-8^-$ precursors from $CD4^+8^+$ products require $p56^{lck}$ activity. In this light, it is intriguing that overexpression of wild-type $p56^{lck}$ in transgenic mice yields thymic tumors comprising cells with an immature phenotype (Abraham *et al.*, 1991c). Hence $p56^{lck}$ may control thymocyte development simply by regulating mitogenesis of T cell progenitors.

Control of T cell receptor gene rearrangement by $p56^{lck}$

We have previously demonstrated that overexpression of wild-type $p56^{lck}$ disrupts T cell receptor expression by specifically arresting Vβ–Dβ joining, without interfering with the rearrangement of other T cell receptor gene segments, including Vα (Anderson *et al.*, 1992). These results suggested that $p56^{lck}$ might ordinarily serve as part of a sensor mechanism that blocks further β gene segment rearrangement once a functional product has appeared. Overexpression of $p56^{lckR273}$ exerted a quite different but equally specific effect. Here both Dβ–Jβ joining and Vβ–Dβ joining occurred at levels comparable to those observed in $CD4^-8^-$ cells from normal mice, however, Vα–Jα gene rearrangements were undetectable. Since α chain gene rearrangement ordinarily follows β gene assembly, this result may simply demonstrate that the developmental blockade resulting from *lck*R273 transcript expression is exquisitely discrete. Alternatively, if one presumes that the sensing mechanism responsible for extinguishing β chain gene rearrangement simultaneously stimulates α chain gene rearrangement (data exists to support this sort of regulatory scheme in the control of antibody gene rearrangements; Alt *et al.*, 1987), then the failure to transduce a $p56^{lck}$-dependent signal might prevent the opening of the α locus that is proposed to permit recombinase access (Diamond *et al.*, 1989; Schlissel and Baltimore, 1989; Ferrier *et al.*, 1990). It should, however, be noted that satisfactory Vδ rearrangements do occur and Vα and Vδ gene segments are intermingled (Chien *et al.*, 1987). It also seems unlikely that the rearrangement patterns observed reflect β rearrangement in γ/δ cells since Vβ–Dβ rearrangements are not generally observed in this lineage (Marusic-Galesic *et al.*, 1988; Raulet *et al.*, 1991).

Regardless of the specific mechanism, failure to produce T cell receptor α chains provides a simple explanation for the near total lack of TCRαβ-bearing cells, since assembly of the T cell receptor complex requires, in most cases, both chains of the heterodimer (Clevers *et al.*, 1988). The absence of α chains cannot, however, explain the *lck*R273-induced reduction in thymocyte number or the absence of $CD4^+8^+$ cells, because animals lacking a functional Cα gene produce $CD4^+CD8^+$ cells at high levels (Philpott *et al.*, 1992). Hence $p56^{lckR273}$ blocks a maturation step associated with, but not dependent on, T cell receptor α chain gene rearrangement. This can be most simply viewed as failure to progress through a stage in development that is ordinarily achieved following receipt of signals from $p56^{lck}$. Figure 6 demonstrates that this point in development corresponds to a stage that had been previously defined using flow cytometric methods. We infer that maturational events prior to this point can proceed satisfactorily in the absence of $p56^{lck}$ function, but that transit through this maturational checkpoint requires $p56^{lck}$. Intriguingly, the maturational defect observed in *lck*R273 thymocytes resembles that recently described by

Mombaerts *et al.* (1992) in mice bearing a targeted disruption of the T cell receptor β chain gene. This observation, coupled with the fact that augmentation of $p56^{lck}$ activity in developing thymocytes specifically blocks $V\beta-D\beta$ joining (Anderson *et al.*, 1992), suggests that $p56^{lck}$ and the β chain may be functionally coupled.

The use of dominant-negative mutations to dissect signaling pathways

Dominant−negative mutations provide powerful experimental tools (Herskowitz, 1987), however, care must be exercised in interpreting the results of such studies. Our experiments illustrate the conceptual intricacies that arise when comparing dominant−negative and targeted disruption approaches to gene inactivation. In particular, we can compare the results presented here with those obtained by Molina *et al.* (1992) studying mice bearing a targeted disruption of *lck* exon 12. Thymocyte number in the *lck* 'knockout' mice decreased by at least 20-fold, reinforcing the view that the *lck*R273 mutation, which compromises the catalytic activity of $p56^{lck}$, yielded a product that effectively blocked normal gene function. Indeed, the fact that a 1:1 ratio of wild-type and catalytically inactive $p56^{lck}$ induced nearly a 50% reduction in total thymocyte number (Figure 3), argues that the *lck*R273 product competed directly with $p56^{lck}$ in developing thymocytes. As the ratio of mutant to wild-type kinase exceeded unity, animals were produced that mimic what would be observed in mice bearing homozygous hypomorphic mutations at the *lck* locus.

Mice bearing dominant−negative *lck* transgenes also differ from lck^{null} animals in important and revealing ways. The lck^{null} mice contain larger numbers of thymocytes, and of $CD4^+8^+$ thymocytes in particular, than do animals from the most severely affected *lck*R273 line, reaching values observed in the 10265 founder animal (Table I). The general pattern of effects measured using these parameters is broadly similar for both the dominant−negative and the recessive *lck* phenotypes; the animals differ primarily in the severity of the defect. Nevertheless, there exist qualitative differences in the phenotypes of the lck^{null} and *lck*R273 mice. For example, virtually all thymocytes in the lck^{null} animals expressed some level of CD3ϵ on their surfaces and >50% were shown to be $CD4^+8^+$ (Molina *et al.*, 1992). Cells with this phenotype were vanishingly rare in thymuses from severely affected *lck*R273 mice. Excluding trivial anomalies that might, for example, result from the use of subtly different mouse strain combinations, four general hypotheses could explain the distinct phenotypes observed in these animals.

First, since the *lck* gene disruption of Molina *et al.* (1992) was generated by manipulating the sequence of the most 3′ coding exon, it is possible that a small amount of functional kinase was produced from the mutant transcript. Although this explanation cannot be excluded completely (save through generation of mice bearing a more definitive *lck* gene disruption), the data of Molina *et al.* indicate that levels of $p56^{lck}$ in thymocytes of their lck^{null} mice were reduced by >95%.

Secondly, it is conceivable that the absence of $p56^{lck}$ in the lck^{null} animals permitted other protein tyrosine kinases to partially assume the normal function of this molecule. Such an effect might not represent true 'redundancy', but instead could describe the limited ability of another possibly very different signaling molecule to promote thymopoiesis, even though this signaling structure may not ordinarily participate in regulating T cell development. If, in the dominant negative case, $p56^{lckR273}$ protein binds and inactivates downstream components of a $p56^{lck}$-dependent signaling pathway, alternative signaling molecules might not easily gain access.

A third explanation for the difference between the lck^{null} and *lck*R273 animals relies on the suggestion that a catalytically inactive protein tyrosine kinase might influence pathways to which the wild-type protein does not normally contribute. These effects, while perhaps illegitimate, are nevertheless specialized and circumscribed. For example, overexpression of a catalytically inactive version of $p59^{fyn}$ under the control of the proximal *lck* promoter disrupted T cell receptor-induced signaling without affecting thymocyte development (Cooke *et al.*, 1991). Subsequent disruption of the *fyn* gene yielded mice with a nearly identical phenotype (Appleby *et al.*, 1992; Stein *et al.*, 1992). Since this closely related *src*-family kinase, overexpressed in catalytically inactive form, did not appear to impinge indiscriminately upon thymocyte regulatory pathways, the attribution of all *lck*R273 transgene effects to rigorously $p56^{lck}$-dependent pathways remains viable.

Finally, it is possible that $p56^{lck}$ exerts effects on thymocyte development that are independent of its kinase activity. Precedent exists for this line of reasoning in the case of the *Drosophila abl* gene, where reconstitution of some *abl*-encoded functions could be achieved in abl^{null} flies using an *abl* gene encoding a catalytically inactive protein (Henkemeyer *et al.*, 1990). Hence the ability of $p56^{lckR273}$ to affect thymocyte development directly and in a positive fashion must be considered.

Functional repertoires of non-receptor protein tyrosine kinases

At least two very similar non-receptor protein tyrosine kinases, $p56^{lck}$ and $p59^{fyn}$, contribute to the regulation of thymocyte function. However, these two closely related kinases differ remarkably in their patterns of expression and in the signaling pathways to which they contribute. In earlier studies, we observed that $p59^{fyn}$ expression begins late in the thymic maturation sequence and that disruption of $p59^{fyn}$-derived signals, achieved either by targeted disruption of the *fyn* gene or by overexpression of catalytically inactive $p59^{fyn}$, compromised delivery of proliferation-inducing signals from the T cell antigen receptor complex (Cooke *et al.*, 1991; Appleby *et al.*, 1992; Stein *et al.*, 1992). Importantly, *fyn*-deficient animals manifested no significant disturbances in thymocyte development. In contrast, overexpression of catalytically inactive $p56^{lck}$ under the control of the same promoter element proved catastrophic to normal thymocyte production. These results strongly suggest that $p56^{lck}$ and $p59^{fyn}$, and by inference *src*-family kinases in general, deliver specialized and non-overlapping signals, even when simultaneously expressed in the same cell type.

Our results support the use of dominant−negative transgenes as tools for the molecular dissection of signaling pathways. This strategy should prove especially powerful in cases where gene disruption yields inviable embryos. For example, the *lck* proximal promoter can direct high level expression of many transgenes to thymocytes. In the worst case, lethal transcripts entrained by the *lck* proximal promoter will provoke thymic agenesis, a defect, which as

shown here in the mouse, yields few systemic consequences. While production of recessive defective mutations in mice can now be achieved (albeit with some difficulty), comparison of the phenotypes of *lck*R273 mice with those of the previously reported *lck*^{null} animals demonstrates that these two approaches together provide complementary insights.

Lastly, we note that the extraordinary sensitivity of thymocyte development to levels of p56^{lck} suggests that heterozygous mutations at the *lck* locus could compromise immune function in humans. Point mutations yielding dysfunctional p56^{lck} molecules will inevitably occur at some frequency and should reduce the effectiveness of p56^{lck} by at least 50%. If such mutations permit expression of inactive protein, thymopoiesis would diminish. This inference, coupled with other studies demonstrating a decline in responsiveness of T cells expressing reduced levels of p56^{lck} (Straus and Weiss, 1992), argues that *lck* mutations may underlie some forms of primary immunodeficiency disease.

Materials and methods

Transgene expression vector and transgenic mouse production

The pLGY(−240) expression vector has been described elsewhere (Allen *et al.*, 1992). A PCR-based mutagenesis strategy was used to convert the codon for Lys273 to an arginine codon as previously described by Hemsley *et al.* (1989). The mutagenesis was carried out on a 2.1 kb *lck* genomic fragment that encompassed exons 3−8. After confirming the presence of the mutation and the absence of other changes by DNA sequencing (Sanger *et al.*, 1977), a 560 bp *Nco*I−*Sal*I fragment containing the mutation was cut out and substituted for the corresponding wild-type fragment in pLGY(−240) (Allen *et al.*, 1992). A 10.5 kb *Xho*I−*Not*I fragment of the assembled vector was purified on low melting point agarose gels and injected into C57BL/6J × DBA/2 F2 zygotes to generate transgenic mice. Transgene-positive animals were identified by hybridization to a probe derived from the human growth hormone 3′ region (Abraham *et al.*, 1991b). Transgenic lines were created by crossing founders to C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME).

Flow cytometry

Lymphocyte suspensions were generated by compression of lymphoid organs between ground glass slides. Red blood cells were lysed using NH_4Cl lysis as previously described by Mishell and Shiigi (1980). Lymphocytes recovered were counted on a hemocytometer and stained for surface expression of CD4 (PE-conjugated GK1.5; Becton Dickinson), CD8 (FITC-conjugated 53-6.71; Becton Dickinson), CD3ε (biotinylated 500AA2; Havran *et al.*, 1987 or FITC-conjugated 145-2C11; Leo *et al.*, 1987), Interleukin-2 receptor (biotinylated PC61; Ceredig *et al.*, 1985), Thy 1.2 (biotinylated 30-H12; Becton Dickinson, T cell receptor Cβ (FITC-conjugated H57.597; Kubo *et al.*, 1989), T cell receptor δ (PE-conjugated GL3; Pharmingen, San Diego, CA), HSA (biotinylated J11D; kindly provided by Dr David Lewis) or CD44 (pgp1; also provided by Dr Lewis). Detection of biotinylated antibodies was facilitated by PE-conjugated streptavidin (Caltag Laboratories, San Francisco, CA). Unconjugated pgp1 antibody was detected using a PE-conjugated goat anti-rat antibody (Caltag Laboratoires). Multi-parameter flow cytometric analysis was carried out on a FACSCAN cell sorter (Becton Dickinson).

Immunoblot analysis

Whole cell lysates were generated by solubilizing thymocytes in TNT buffer (150 mM NaCl, 50 mM Tris−Cl pH 8, 1% Triton X-100, 1 mM $NaVO_4$ and 1 mM PMSF) for 20 min at 4°C. Insoluble material was removed by centrifuging at 12 000 *g* for 5 min at 4°C. Lysates were then boiled in SDS sample buffer, resolved on 10% SDS−PAGE and transferred to nitrocellulose. The filter was blocked in 5% milk−0.1% Tween-20. A rabbit polyclonal antiserum specific for p56^{lck} (195.7, Louie *et al.*, 1988) was used at a concentration of 1:5000 in 1% milk and 0.1% Tween-20. Following a 2 h incubation at room temperature, the filters were rinsed in 1% milk−0.1% Tween-20 and developed using a donkey anti-rabbit secondary antibody and the Enhanced Chemiluminescence detection system (Amersham). Total p56^{lck}was quantitated using scanning densitometry and expressed relative to levels of endogenous p56^{lck} as determined from extracts of normal thymocytes on the same autoradiogram.

Cell cycle analysis

DNA content was determined for thymocytes by a modification of the procedure of Krishan (1975). Briefly, cells were stained for CD4 and CD8 as above and fixed in paraformaldehyde. The fixative was removed and nuclei treated with propidium iodide solution and RNAase as described (Krishan, 1975). Flow cytometry was carried out on a FACSCAN cell sorter (Becton Dickinson) using Lysis II software (Becton Dickinson).

Detection of rearrangement by PCR

PCR-based analysis of β-chain rearrangement was performed using an assay modified from that described by van Meewijk *et al.* (1990). High molecular weight thymocyte DNA was extracted (1 × phenol, 2 × phenol−chloroform, 1:1), ethanol-precipitated and resuspended in TE (10 mM Tris−HCl pH 7.5 and 1 mM EDTA) prior to quantitation and use. PCR amplifications were performed in 50 μl reaction buffer (50 mM KCl, 20 mM Tris−HCl pH 8.3, 2.5 mM $MgCl_2$ and 0.1 mg per ml BSA) containing 0.5 μg template DNA, 1 μM 5′ and 3′ primers, 0.2 mM each dNTP (Pharmacia) and 1 U AmpliTaq DNA polymerase (Perkin−Elmer Cetus). The amplification cycle (1 min at 95°C, 2 min at 63°C and 10 min at 72°C) was repeated 24 times. 12.5 μl of the PCR reaction were fractionated on a 1.8% agarose gel, blot transferred and the filters hybridized with a Jβ2-specific oligonucleotide probe located upstream of the 3′ PCR primer (Malissen *et al.*, 1984). Rearrangements to individual Jβ elements can be visualized by this assay.

Vα−Jα rearrangements were detected using the same PCR strategy, using a pool of 5′ primers specific for three Vα elements and 3′ primers specific for individual Jα segments (Hayday *et al.*, 1985; Winoto *et al.*, 1985; Chou *et al.*, 1986). The amplification cycle (1.5 min at 95°C, 2 min at 63°C and 3.5 min at 72°C) was repeated 24 times and products were detected with Jα specific oligonucleotide probes located upstream of the 3′ PCR primer.

The following synthetic oligonucleotides were used as primer for PCR amplification or as hybridization probes:

Vβ12-5′	5′-AGTTACCCAGACACCCAGACATGA-3′
Jβ2-3′	5′-TGAGAGCTGTCTCCTACTATCGATT-3′
Vα5H-5′	5′-CAGAAGGTGCAGCAGAGCCCAGAA-3′
VαF3-5′	5′ACCCAGACAGAAGGCCTGGTCACT-3′
Vα2C-5′	5′-ACTGTCTCTGAAGGAGCCTCTCTG-3′
JαTT11-3′	5′-GACCCTATTACTCACATACTTGGCTTG-3′
Jβ2-Probe	5′-TTTCCCTCCCGGAGATTCCCTAA-3′
JαTT11-Probe	5′-GAAAGCAGAGTCCCAATTCCAAAG-3′

Generation of normal double−negative cells

Normal double−negative cells were generated using antibodies to CD4 (GK1.5 ascites) and CD8 (53-6.71 ascites) and complement lysis as described by Lewis *et al.* (1988). Cells were 97% pure as judged by subsequent CD4 and CD8 staining.

Quantitation of mRNA levels by Northern and slot blots

RNA was prepared according to the method of Chomczynski and Sacchi (1987) and resolved on 1% agarose−formaldehyde gels (Allen *et al.*, 1992) or subjected to slot blotting using an apparatus from Schleicher and Schuell under conditions recommended by the supplier. Blots were incubated with random-primed probes generated from human growth hormone sequences, an *lck* cDNA, or a mouse EF-1α cDNA as described by Allen *et al.* (1992). Quantitation was performed using scanning densitometry (Abraham *et al.*, 1991c,b).

Acknowledgements

We thank Joan Carpenter and Jill Thomas for skilful assistance in maintaining our animal colony, David Coder and Kerry Ryan for help with cell cycle analysis, Dr Richard Palmiter for reading an early draft of the manuscript, Kathi Prewitt for help in assembling the manuscript, and our colleagues for helpful criticisms. S.D.L. was supported by an Immunology Training Grant from the National Cancer Institute (CA09537). This work was partially supported by the NIH (CA04682) and by a generous gift from the Pfizer Corporation to the Department of Immunology, University of Washington.

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Received on November 24, 1992; accepted on January 19, 1993