The Protein Tyrosine p56^{lck} Regulates Thymocyte Development Independently of Its Interaction with CD4 and CD8 Coreceptors

By Steven D. Levin,*‡ Kristin M. Abraham,‡ Steven J. Anderson,*§ Katherine A. Forbush,*§ and Roger M. Perlmutter*‡§‖

From the *Howard Hughes Medical Institute; and the Departments of ‡Biochemistry, §Immunology, and ‖Medicine (Medical Genetics), University of Washington, Seattle, Washington 98195

Summary

The *lck* gene encodes a lymphocyte-specific protein tyrosine kinase of the nonreceptor type that is implicated in signal transduction pathways emanating from the CD4 and CD8 coreceptors. Previous studies also support a role for p56^{lck} in regulating T cell receptor β gene rearrangements and, more generally, thymocyte development. Here we report that a mutant form of p56^{lck}, which is incapable of interacting with CD4 or CD8, behaves indistinguishably from association-competent p56^{lck} with respect to its ability to affect thymocyte maturation. The effects of p56^{lck} remained specific in that the closely related *src*-family kinase p59^{hck} was incapable of substituting for p56^{lck} in arresting β locus gene rearrangements. These data support the view that *src*-family kinases perform highly specialized and often nonoverlapping functions in hematopoietic cells, and that p56^{lck} acts independently of its association with CD4 and CD8 to regulate thymocyte development.

The p56^{lck} signaling molecule is a 509 residue *src*-like protein tyrosine kinase that is expressed almost exclusively in T lymphocytes (1–3). Originally identified as the product of a gene that was activated by retroviral insertion in certain murine lymphoid cell lines (1, 4), the *lck* gene is expressed in thymocytes from the time that hematopoietic progenitors first colonize the thymic anlage, and *lck* transcripts accumulate in all thymocyte subsets and all mature T cell populations (1, 2, 5–7). Involvement of p56^{lck} in lymphocyte signaling was suggested by the ability of mutant activated forms of this protein, those lacking a conserved COOH-terminal tyrosine phosphorylation site (Y505), to transform both fibroblasts in vitro (8, 9) and lymphoid cells in transgenic mice (10). Moreover, p56^{lck} physically associates with the CD4 and CD8 coreceptor structures on T lymphocytes (11–13), and becomes activated when CD4 is crosslinked using anti-CD4 antibodies (14–16). Paired cysteine residues in the NH$_2$-terminal domain of p56^{lck}, and in the short intracellular segments of both CD4 and CD8, are required for this association (17, 18). In one study, transfection of a lysozyme-specific T cell hybridoma that requires CD4 for antigen recognition with constructs encoding a CD4 molecule lacking these cysteine residues resulted in loss of CD4 signaling capacity (19). These observations, in aggregate, have given weight to the hypothesis that antigen recognition by T cells is potentiated when the CD4 or CD8 coreceptor structure becomes associated with the antigen receptor complex, thereby approximating the p56^{lck} kinase to the CD3 signaling machinery (20).

Whereas p56^{lck} almost certainly serves a crucial function in relaying signals from coreceptor molecules, there are many circumstances in which the signaling capacity of p56^{lck} appears unrelated to CD4 and CD8 expression. For example, p56^{lck} is expressed at high levels in NK cells (2, 6, 21, 22), where CD4 and CD8 do not participate in antigen recognition. Moreover, *lck* transcripts accumulate to the greatest extent in thymocytes, including CD4$^-$8$^-$ thymocytes (2, 5–7). In one set of experiments, expression of activated p56^{lck} (bearing a phenylalanine residue at position 505) in an insulin-reactive T cell line yielded cells with a much increased sensitivity to antigen, despite the fact that these cells failed to express CD4 or CD8 (23). A more recent study demonstrated that loss of p56^{lck} expression in the CD4$^-$8$^-$ transformed human T cell line Jurkat resulted in a nearly complete block in TCR-mediated signaling which could be reconstituted by expression of wild-type p56^{lck} (24). These results suggest that p56^{lck} may contribute to T cell signaling pathways other than those that emanate from the CD4 and CD8 receptors. Consistent with this view, p56^{lck} has been shown to associate with the β chain of the IL-2 receptor, and becomes activated after IL-2 treatment of normal T cells (25, 26).

Previous studies demonstrate that thymocyte development

is exquisitely sensitive to levels of p56^{lck} activity. Mice bearing a targeted disruption of the *lck* gene (27), or expressing high levels of a dominant-negative form of p56^{lck} (28), manifest a severe block in thymocyte maturation resulting in a 20–50-fold reduction in thymocyte number, primarily as a result of decreased generation of CD4$^+$8$^+$ cells from CD4$^-$8$^-$ precursors.

Augmented expression of p56^{lck} in transgenic mice under the control of its own promoter provided more insight into this phenomenon. Mice expressing very high levels of p56^{lck}, and especially those expressing the mutant (F505) activated form, rapidly develop thymic tumors, supporting the view that p56^{lck} activity promotes mitogenesis (10). More importantly, even modest overexpression of p56^{lck} yields thymocytes in which TCR gene rearrangement no longer follows the normal developmental sequence. In these animals, joining of Vβ gene segments to Dβ gene segments fails to occur, even though Vα to Jα rearrangement, ordinarily a later event, proceeds normally (29, 30). These observations support a model in which p56^{lck} serves to regulate rearrangement at the β locus, perhaps by perceiving the correct expression of a Cβ-containing gene product (30).

In principle, the signaling disturbances that occur in mice expressing high levels of p56^{lck} could reflect delivery of signals from the CD4/CD8 coreceptors, from the IL-2 receptor, or from some other receptor structure. To elucidate the nature of this signaling pathway, we have generated transgenic mice in which the same construct previously employed to express high levels of p56^{lck} in thymocytes was modified to permit expression of a mutant form of p56^{lck} that cannot interact with CD4 or CD8, and have examined the effects of simultaneous overexpression of wild-type CD4 in both animal systems. We have also expressed an activated version of a closely related protein tyrosine kinase, p59^{hck}, in thymocytes using the same thymocyte-specific promoter. Examination of these animals reveals that interaction with CD4 is not required to permit elaboration of an *lck*-overexpression phenotype, and indeed CD4 actually appears to "sequester" p56^{lck} and render it less potent. Our results provide strong support for the proposition that p56^{lck} regulates thymocyte development independently of its ability to interact with CD4 and CD8, and does so uniquely, in that closely related kinases, expressed in activated forms and at high levels, cannot mimic its effects.

Materials and Methods

Transgene Expression Vector and Transgenic Mouse Production. The p*lck*F505 expression vector has been described elsewhere (10, 29). Site-directed mutagenesis (31) was used to convert cysteine codons 20 and 23 to alanine codons (codon 20 changed from TGT to GCC and codon 23 changed from TGC to GCG). The mutagenesis was carried out on a *lck* cDNA. After confirming the presence of the mutation and the absence of other changes by DNA sequencing (32), a 125-bp StuI/BglII fragment containing the mutation was isolated and substituted for the corresponding wild-type fragment in p*lck*F505 (29). An 11.2-kb XhoI/NotI fragment was then purified

on low melting point agarose gels and injected into C57BL/6J × DBA/2 F$_2$ zygotes to generate transgenic mice.

The p1017 vector has been described (33, 34), as has a human *hck* cDNA in which site-directed mutagenesis was employed to convert tyrosine codon 501 to a phenylalanine codon (35). The *hck*F501 cDNA was purified as an EcoRI fragment, made blunt-ended using T4 DNA polymerase, and ligated into p1017 at the BamHI site which was also filled-in using T4 DNA polymerase. A 7-kb NotI fragment was excised and used to generate transgenic mice as above.

Transgene-bearing animals were identified by hybridization to a probe for the human growth hormone 3' region (10, 29). Transgenic lines were created by crossing founders to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME).

Flow Cytometry. Lymphocyte suspensions were generated by compression of lymphoid organs between ground glass slides. RBC were lysed using NH$_4$Cl lysis as previously described (29). Lymphocytes recovered were counted on a hemocytometer and stained for surface expression of CD4 (PE-conjugated GK1.5; Becton Dickinson & Co., Mountain View, CA), CD8 (FITC-conjugated 53-6.71; Becton Dickinson & Co.), CD3ϵ (biotinylated 500AA2, reference 36; or FITC-conjugated 145-2C11, reference 37), or Thy 1.2 (biotinylated 30-H12; Becton Dickinson & Co.). Detection of biotinylated antibodies was facilitated by PE-conjugated streptavidin (Caltag Laboratories, San Francisco, CA). Multi-parameter flow cytometric analysis was carried out on a FACScan® cell sorter (Becton Dickinson & Co.).

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$_4$, 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% (p56^{lck}) or 12% (p59^{hck}) SDS-PAGE, and transferred to nitrocellulose. For p56^{lck} and p59^{hck} detection, filters were blocked in 5% milk, 0.1% Tween 20. Rabbit polyclonal antisera specific for p56^{lck} (195.7; 38) or p59^{hck} (6168.2; 35) were used at a concentration of 1:5,000 in 1% milk, 0.1% Tween 20. After 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 Chemiluminesence detection system (Amersham Corp., Arlington Heights, IL).

Immunoprecipitations and In Vitro Kinase Assay. Immunoprecipitations of kinases were performed using the same antisera described above at a 1:200 dilution from 10–100 µg of total thymocyte protein. Anti-CD4 immunoprecipitates were performed using the mAb GK1.5 plus a rabbit anti–rat IgG secondary antibody. Immune complexes were collected using Pansorbin (Calbiochem-Novabiochem, La Jolla, CA). Pellets were washed five times in TNT and two times in high salt TNT (1 M NaCl), and resuspended in kinase buffer (20 mM Tris-Cl [pH, 7.5], 10 mM MnCl$_2$, 1 mM Na$_2$VO$_4$, 0.1% Triton X-100, plus 2 µl per reaction of γ-[^{32}P]ATP (3,000 Ci/mmol). These reactions were allowed to proceed for 10 min at room temperature, and were stopped by addition of SDS sample buffer and by boiling for 3 min before resolution on SDS-PAGE. Gels were dried and exposed to X-ray film. ^{32}P incorporation into protein bands was determined on a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA).

Quantitation of mRNA Levels by Northern and Slot Blots. RNA was prepared according to the method of Chomczynski and Sacchi (39) and resolved on 1% agarose formaldehyde gels (40) or examined using a slot blotting apparatus (Schleicher & Schuell, Inc., Keene, NH). Blots were probed with ^{32}P-labeled probes generated from human growth hormone sequences, from *lck* or *hck* cDNA,

or from a mouse EF-1α cDNA as described (40). Quantitation was performed using scanning densitometry (10, 29).

Detection of β Chain Rearrangements by PCR and Northern Blot. The synthetic oligonucleotides, PCR amplification, gel transfer, probes, and blot hybridization conditions were performed as described previously (30). Northern blots were prepared as described above, but were probed with ^{32}P-labeled Cβ or Cα probes as described (30).

Results

Expression of a CD4/CD8 Association-Defective lckF505 Transgene. To investigate the possibility that p56^{lck} might act independently of CD4 and CD8 in blocking thymocyte maturation, we constructed a transgene that was identical to the lckF505 transgene employed previously (10, 29; Fig. 1 *A*), but which also had alanine codons substituted for cysteine codons 20 and 23 (Fig. 1 *B*). Substitution of a phenylalanine codon for tyrosine at position 505 yields an "activated" version of p56^{lck} that exhibits a 3–7-fold greater potency than does its wild-type counterpart (10, 29). Previous work has demonstrated that the alanine substitutions at positions 20 and 23 eliminate the ability of p56^{lck} to couple to CD4 and CD8 (17 and see below). Hence the lckF505A20,23 transgene product should be activated, by virtue of the F505 mutation, but unable to interact with CD4 or CD8, because of the A20/A23 substitutions. This transgene should therefore provide information regarding CD4/CD8-dependent and -independent lckF505 effects.

In all, twelve founder animals and six mouse lines were

Figure 1. Transgene expression vectors. (*A*) Structure of the lckF505 transgene. Exons are denoted by numbered boxes. The positions of the translation initiation and termination codons are also noted (10, 29). (*B*) Structure of the lckF505A20,23 transgene. This construct differs from lckF505 (*A*) only in that alanine codons have been substituted for cysteine codons 20 and 23. (*C*) Structure of the p1017hckF501 transgene. See text for description.

analyzed for transgene expression and thymocyte cell surface phenotype (Table 1). Transgene expression was limited to the thymus and mRNA level were proportional to the number of copies integrated into the genome (data not shown), as was observed with the previous lckF505 construct (10, 29). Mice that expressed the lckF505A20,23 transgene at roughly twofold over endogenous lck mRNA levels developed thymic tumors (Table 1) with approximately the same kinetics as lckF505 animals (10). It is interesting that progeny of the 7121 and 7148 founder animals developed tumors sporadically (Table 1), suggesting that their expression levels lie near the threshold for tumor development (10). All tumors were phenotypically immature as judged by CD3, CD4, and CD8 staining (Fig. 2) and were indistinguishable from tumors produced in lckF505 mice (10). We conclude from this that transforming potential of p56^{lck} is independent of its ability to associate with CD4 and CD8.

In lckF505A20,23 mice that did not develop tumors, a defect in thymocyte maturation was observed that reflected an inability to produce CD3-bearing CD4$^+$8$^-$ and CD4$^-$8$^+$ (single positive) cells (Fig. 3) although total thymocyte numbers were unchanged (data not shown). Instead, increased percentages of CD4$^+$8$^+$, CD4$^-$8$^-$, and CD8$^+$/CD3$^-$ cells (believed to represent precursors to CD4$^+$8$^+$ cells) were observed. Construction of a dose–response curve plotting transgene expression versus the number of CD3$^+$ cells demonstrated that the lckF505 and lckF505A20,23 transgenes exhibit identical characteristics (Fig. 4). Peripheral T cell numbers were also reduced in lckF505A20,23 mice, again in a manner that was indistinguishable from lckF505 animals (Table 1 and data not shown).

The inability to generate CD3$^+$ cells in lckF505 mice results primarily from a failure to rearrange Vβ TCR gene segments, such that thymocytes from these animals contain 1.0-kb β locus transcripts that result from Dβ-Jβ joining, but lack mature 1.3-kb products that ordinarily reflect Vβ-Dβ rearrangement (29, 30). Fig. 5 demonstrates that this defect also characterizes thymocytes from lckF505A20,23 mice. Thus mature 1.3-kb β chain transcripts are not observed in developing thymocytes from lck transgenic animals, but the 1.0-kb Dβ-Jβ mRNA is readily detected (Fig. 5 *A*). Quantitation of specific Vβ to Dβ-Jβ rearrangements by PCR amplification of thymocyte DNA confirms a reduction in joining events comparable to that seen in lckF505 mice (Fig. 5 *B*). Dβ to Jβ joining events, however, were detected at levels comparable to those in normal thymocytes (Fig. 5 *B*). Fig. 5 *A* also shows that full-length α chain mRNA is present at normal levels in thymocytes from both types of transgenic mice. We conclude that a lck transgene product which was presumably unable to interact with either CD4 or CD8 produced the same developmental abnormalities, apparently by a similar mechanism, as a transgene product which could physically couple to these coreceptors.

The lckF505A20,23 Transgene Product Does Not Immunoprecipitate with CD4. One possible explanation for our observations might be that p56^{lckF505A20,23} can in fact interact with the CD4 and CD8 coreceptors under certain circum-
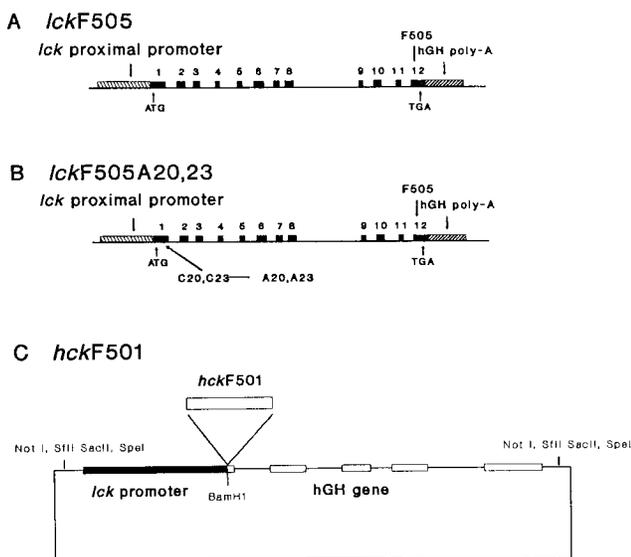
Table 1. *Summary of Results Obtained from lckF505A20,23 Transgenic Mice*

Line	Number	Expression	Tumor Formation	CD3$^+$ (Thymus)	CD3$^+$ (Periphery)
7137	1	ND	+	31.0	13
4965	1	51.6	+	13.6	15
5044	2	29.3	+	12.0	15
5049	1	29.1	+	1.0	15
7178	1	26.5	+	23.6	57
7121	7	23.6	± [*]	10.4	20
7148	6	20.7	± [‡]	9.8	22
7120	2	11.6	−	6.2	50
4966	1	6.8	−	36.5	62
5057	1	5.2	−	55.0	60
5048	1	4.9	−	59.3	78
5045	4	1.5	−	101	95
4972	6	0.9	−	102	110
4967	1	0.6	−	109	86

Transgene expression levels are given as pg of transgene mRNA/μg total RNA. The mean number of CD3$^+$ thymocytes and peripheral T cells is presented (as percentage of control values) for each line examined. Tumor formation was scored by visual inspection and increased forward light scatter (indicative of an increase in thymocyte size which typically accompanies transformation).
[*] Two out of seven animals developed tumors in this line.
[‡] One out of six animals developed a tumor in this line.

stances. For example, our previous experiments, and those of others, defining the region of p56^{lck} responsible for coreceptor binding were performed under nonphysiological conditions in fibroblast cell lines (17, 18). To address the extent of association of p56^{lckF505A20,23} with CD4 in lymphoid cells, we performed immunoprecipitation experiments using thymocyte extracts from normal, lckF505, and lckF505A20,23 mice. Anti-p56^{lck} immunoprecipitates showed elevated kinase activity in both lckF505 and lckF505A20,23 extracts, consistent with overexpression of the transgenes and the enhanced activity associated with the F505 mutation (Fig. 6). However, in anti-CD4 immunoprecipitates elevated kinase activity was observed only in the lckF505 lysates. The lckF505A20,23 extracts contained CD4-associated kinase activity at about the levels seen in normal animals (Fig. 6). This suggested that only endogenous wild-type p56^{lck} was coupling with CD4 in lckF505A20,23 lysates in this in vitro assay.

Rescue of lckF505 Transgene Effects by Expression of a CD4 Transgene. Additional in vivo experiments permitted a much more persuasive test of the interaction of p56^{lckF505A20,23} with CD4 and provided further evidence of the coreceptor-independent nature of the lck transgene effects. Previously we reported the generation of lines of transgenic mice that express high levels of mouse CD4 under the control of the lck proximal promoter (41). If overexpression of p56^{lck} interferes with thymocyte development through overzealous delivery of a coreceptor-derived signal, we reasoned that simultaneous overexpression of CD4 and lck might yield additive

effects. We therefore crossed lckF505 transgenic mice with lck-CD4 mice (line 727), the thymocytes of which all express very high levels of CD4 (about 15-fold over normal CD4 levels seen in CD4$^+$8$^+$ cells). The lck-CD4 transgenic mice normally exhibit no defects in TCR gene rearrangements (data not shown), although the level of CD3 expression in immature CD3^{low} cells is increased somewhat (41).

Comparison of the number of CD3$^+$ cells in progeny from this cross revealed a remarkable recovery of CD3$^+$ cells in thymuses from animals carrying both transgenes, as compared with thymuses from animals transgenic for lckF505 alone (Fig. 7). In three lckF505 lines tested, coexpression of the CD4 transgene resulted in recovery of substantial numbers of CD3$^+$ cells. Hence, it appeared that excess CD4 mitigated, to some extent, the effects of the lckF505 transgene, perhaps by binding and sequestering the excess p56^{lck}.

However, it was also possible that signals derived from the overexpressed CD4 product, though essentially undetectable themselves, actually acted independently of p56^{lck} to antagonize the effects of the kinase. This explanation could be excluded by examining the maturation of thymocytes from the lckF505A20,23 mice that also expressed high levels of CD4. If the lckF505A20,23 transgene product was behaving as expected (i.e., not associating with CD4), there should be no recovery of CD3$^+$ cells despite the presence of excess CD4, since sequestration should not occur. Fig. 8 shows that this was in fact the case. No improvement in the representation of CD3-bearing cells was observed in the double-
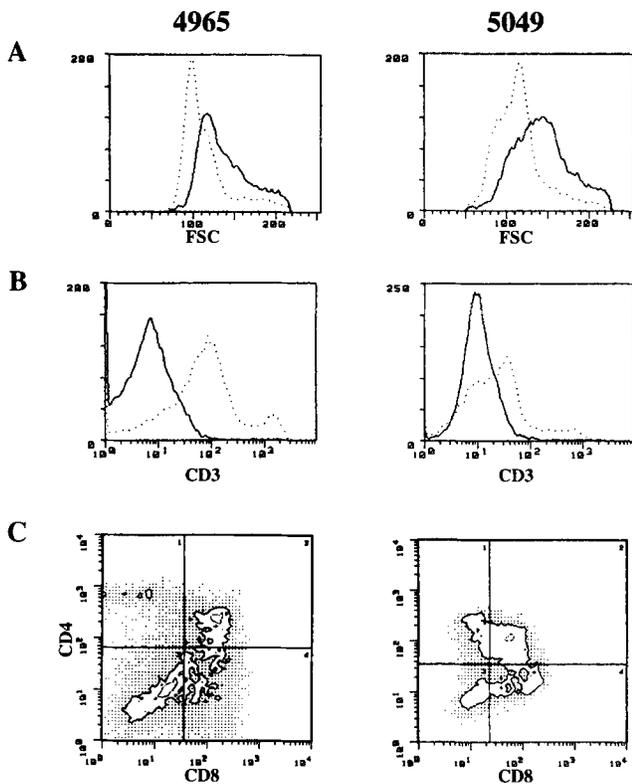
Figure 2. Properties of tumors generated by overexpression of *lck*F505A20,23. Thymocyte flow cytometric profiles from two independent founder animals expressing the *lck*F505A20,23 transgene. These animals had developed grossly visible thymic tumors. (*A* and *B*) (*Solid line*) Cells from the transgenic animal; (*dotted line*) control cells. (*A*) Forward scatter profiles (as a surrogate index for cell size). (*B*) CD3 expression. (*C*) CD4/CD8 dual parameter flow cytometric profiles document increased representation of immature CD4⁻/CD8⁻ cells.

ulocytes (6, 43). We have previously demonstrated that substitution of phenylalanine for tyrosine at position 501 yields an especially potent transforming gene when tested in NIH 3T3 cell transfection assays (35). A *hck* cDNA bearing the appropriate mutation was inserted into the p1017 vector (Fig. 1 *C*) which comprises a 3.2-kb *lck* proximal promoter fragment linked to the human growth hormone (hGH) gene which provides intronic structure, a polyadenylation signal, and a unique 3'-untranslated region (33, 34). With this vector system, transcript levels of the *hck* transgene could be directly compared with those observed with the *lck* transgenes since each incorporates the same 3' untranslated region.

Results obtained through examination of eight independent founder animals and two lines of *hck*F501 animals are summarized in Table 2. Expression of the *hck*F501 transgene failed to produce any tumors or phenotypic abnormalities in developing thymocytes in spite of the fact that transgene mRNA expression levels were, in at least one case, as high as those observed in *lck*F505 tumor-bearing lines (Table 2). There were also no discernible abnormalities in peripheral T cells (Table 2 and data not shown). Flow cytometric analysis revealed patterns of CD4, CD8, and CD3 staining that were indistinguishable from control animals for both thymocytes and mature peripheral T cells (data not shown) in spite of the fact that the *hck* protein, not normally present in thymocytes, was readily detectable in extracts from *hck*F501 thymocytes (Fig. 9). We conclude that simple augmentation of the levels of a *src*-family protein tyrosine kinase in developing thymocytes is not sufficient to provoke either tumorigenesis or developmental perturbations. Hence the effects observed with the *lck* transgenes reflect some unique activity of p56^lck that is independent of its ability to interact with the CD4 and CD8 coreceptors.

transgenic (*lck*F505A20,23 × CD4) mice. The failure to rescue CD3⁺ cells in *lck*F505A20,23 mice with the *lck*-CD4 transgene demonstrates that p56^lckF505A20,23 fails to associate with CD4 in vivo (as expected), and that the attenuation of the effects of the *lck*F505 transgene in mice simultaneously overexpressing CD4 is due to the interaction of p56^lck with CD4, which in this case suppresses, rather than stimulates, a kinase-mediated signaling process.

Specificity of lckF505 Induced Abnormalities. Thymocytes contain numerous protein tyrosine kinases besides p56^lck (2, 6, 42). Since the developmental perturbations induced by *lck* overexpression occurred independently of its association with the CD4/CD8 coreceptors, it was possible that these abnormalities might result from a nonspecific effect of augmented protein tyrosine kinase activity. To investigate this hypothesis, we generated constructs in which a closely related *src*-family kinase, p59^hck, in an activated form lacking the regulatory COOH-terminal tyrosine (*hck*F501), was expressed under the control of the same *lck* proximal promoter element. The *hck* gene product is not normally found in thymocytes, but is present in cells of the myeloid lineage, especially gran-

Discussion

p56^lck: A Unique Regulator of Thymocyte Maturation. The experiments reported above were initiated in an attempt to define the mechanism whereby expression of high levels of p56^lck perturbs the normal thymocyte maturation sequence. Previous studies permitted us to construct a dose–response curve for the effects of augmented p56^lck activity, and revealed that even very modest augmentation of the level of wild-type p56^lck protein, expressed under the control of its own transcriptional regulatory elements, resulted in very substantial developmental abnormalities (29). Subsequent investigation revealed that the most prominent feature of this maturational arrest, the inability to generate CD3⁺ thymocytes, resulted from a single molecular defect: the failure to catalyze Vβ-Dβ joining and hence the inability to produce a functional TCR β chain (30). The exquisitely specific nature of this abnormality suggested that augmentation of p56^lck activity impinged on a normal regulatory process involved in controlling TCR gene rearrangement. However, in light of the fundamental regulatory roles of protein tyrosine kinases generally, it was possible that the developmental block induced by overexpression of p56^lck might be non-
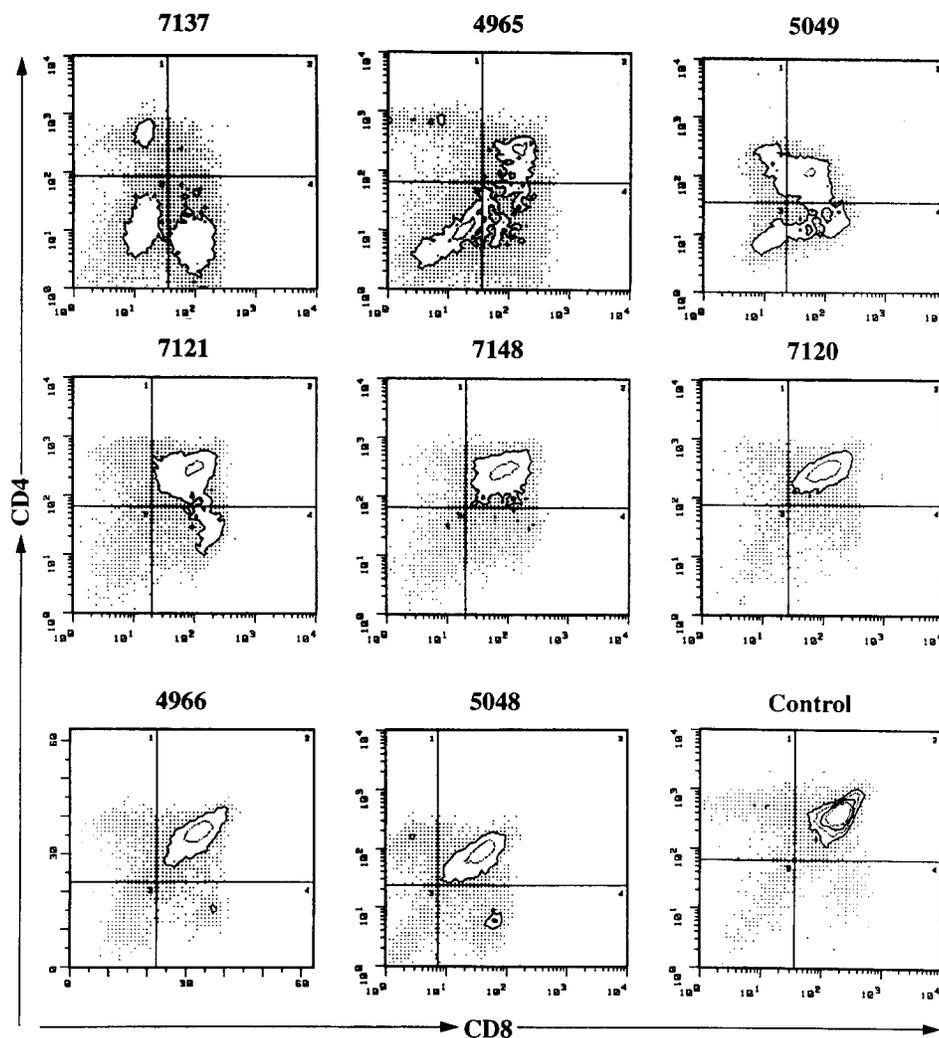
7137   4965   5049

7121   7148   7120

4966   5048   Control

CD4 →

CD8 →

Figure 3. Increased *lck*F505A20,23 transgene expression correlated with the degree of abnormality observed in CD4 × CD8 profiles. Thymocyte CD4 × CD8 two-parameter profiles for representative animals in each of eight lines of mice (see Table 1 for expression values). Profiles are arranged from highest expression level to the lowest. (*Control*) Results of this analysis performed using cells from a control thymus.
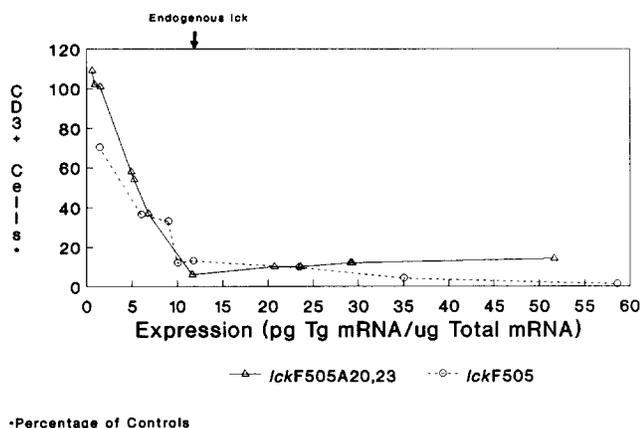

—△— *lck*F505A20,23   ··○·· *lck*F505

•Percentage of Controls

Figure 4. Expression of the *lck*F505A20,23 transgene affected development of CD3+ cells with the same potency as the *lck*F505 transgene. (*x-axis*) Transgene expression is plotted as pg of transgene mRNA per μg of total RNA. (*y-axis*) Number of CD3+ cells (as percentage of control values). Results obtained using animals expressing the *lck*F505A20,23 transgene (-△-) are compared with values previously reported (29) for *lck*F505 mice (-○-). The level of mRNA that corresponds to endogenous *lck* transcript abundance in this assay is indicated (≈12 pg/μg).

specific, the result of hyperstimulation of a regulatory mechanism that need not, under normal circumstances, involve p56^{lck} itself at all.

Whereas we cannot exclude this argument completely, the results reported here, coupled with other studies, serve to focus attention on p56^{lck} as a unique regulator of early thymocyte maturation. First, p56^{lck} is ordinarily expressed at highest levels in immature thymocytes (2, 5–7). This contrasts with other nonreceptor protein tyrosine kinases, notably p59^{fynT}, which are expressed at highest levels in mature T lineage cells (33). Second, although thymocytes are extraordinarily sensitive to the presence of augmented levels of p56^{lck}, simple overexpression of other nonreceptor protein tyrosine kinases does not appreciably alter early thymocyte maturation. For example, overexpression of p59^{fyn} under the control of the proximal *lck* promoter yields animals with T cells that respond inordinately vigorously to stimulation of the TCR, but the thymocytes from these animals mature normally (33). In the present study, we used the same promoter element to express p59^{hck}, a myeloid cell–specific protein tyrosine kinase ordinarily expressed at highest levels in gran-
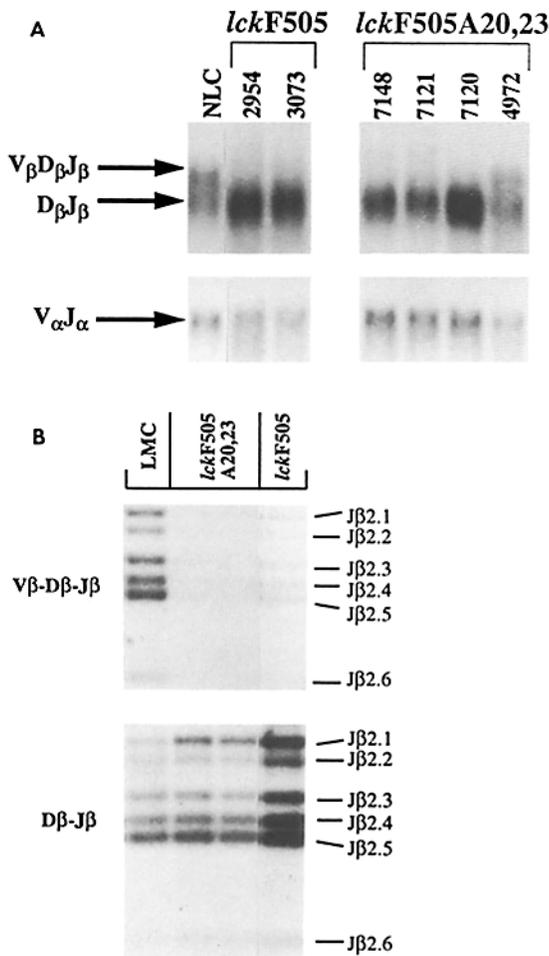
250   p56^{lck} Acts Independently of CD4/CD8 T Cell Development

A

B

Figure 5. Thymocytes from *lck*F505A20,23 mice were deficient in Vβ rearrangements and mature TCR-β transcripts but produced normal levels of TCR-α transcripts. (*A*) Total thymocyte RNA was prepared from a normal mouse (*NLC*), from two lines of *lck*F505 mice, and from four lines of *lck*F505A20,23 animals. 10 μg of each RNA sample was separated on a 1% agarose/formaldehyde gel, blotted onto a nylon filter, and probed for Cβ- (*top*) or Cα- (*bottom*) containing transcripts. Two Cβ transcripts were observed: an immature 1.0-kb form which represents transcription initiating adjacent to rearranged Dβ elements, and the mature 1.3-kb transcript originating from VβDβJβ rearranged DNA. Only the immature transcript was present in all transgenic animals except for the *lck*F505A20,23 4972 line which expressed very little of the transgene mRNA and produced normal numbers of CD3+ cells (see Table 1). A single 1.6-kb mature Cα transcript was observed and this was present at roughly the same levels in all thymocyte RNA samples examined. (*B*) Vβ8 (*top*) and Dβ2 (*bottom*) rearrangements to Jβ2 segments were amplified by PCR using thymocyte DNA from the indicated normal or transgenic animals. Specific rearrangements in transgenic thymocytes are reduced in abundance by >80% as compared with control thymocytes.

ulocytes. In fibroblast transformation assays, p59[hckF501] exhibits 100-fold more transforming activity than does p56[lckF505] (35). Yet, thymocytes tolerate high level expression of this potent transforming gene with impunity (Table 2). Hence p56[kk] acts uniquely in blocking thymocyte maturation.

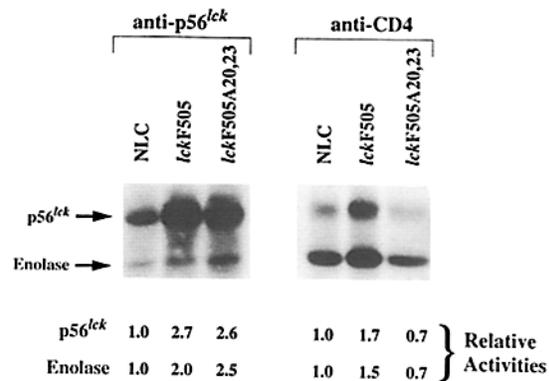It should also be noted that mice lacking a functional *lck*

Figure 6. p56[lckF505A20,23] failed to associate with CD4 in thymocyte extracts. Detergent extracts were prepared (see Materials and Methods) from thymocytes isolated from a normal mouse (*NLC*), from an animal of the *lck*F505 3073 line (29), or from an animal of the *lck*F505A20,23 7120 line. Immunoprecipitations were performed using either an anti-p56[kk] antiserum or an anti-CD4 mAb. In vitro kinase activity was assayed using these immune complexes, and the products were subsequently resolved on 10% SDS-PAGE. Autophosphorylation of p56[kk] and phosphorylation of enolase were measured by quantitation of ^{32}P incorporation using a Molecular Dynamics Phosphorimager. These values (*bottom*) are expressed relative to those obtained using immunoprecipitates from the normal thymocytes.
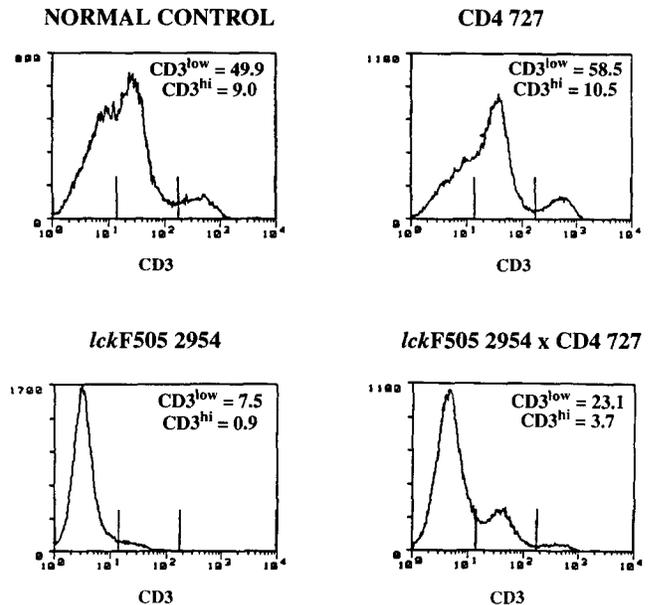

Figure 7. Expression of a CD4 transgene rescued CD3+ thymocyte development in *lck*F505 mice. Animals of the *lck*F505 2954 line (29) were crossed to the *lck*-CD4 727 line (41). Shown here are representative examples of CD3 histograms of thymocytes from progeny of a single cross. CD3 fluorescence is plotted versus relative cell number. The genotype of each animal is shown above the profile and the percentages of cells expressing CD3 at high and low densities are indicated.

gene exhibit a distinct, but nevertheless severe, defect in thymocyte maturation consisting of a marked decrease in total thymocyte number with relative preservation of the most immature (CD4⁻8⁻) cells (27). We have obtained similar results
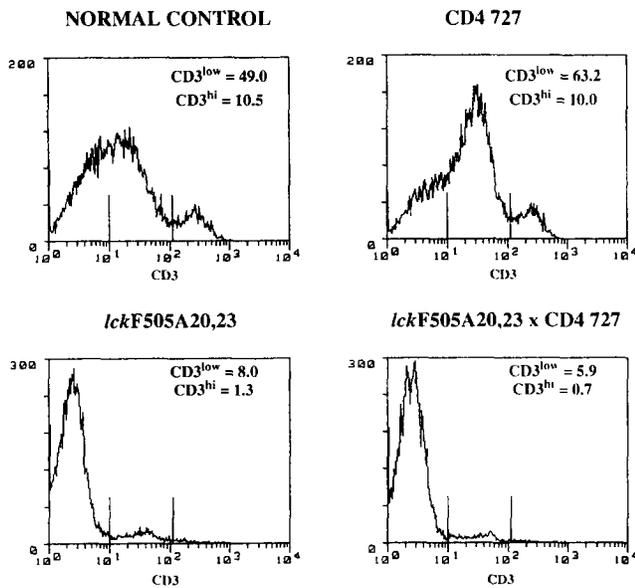
Figure 8. Expression of a CD4 transgene failed to rescue CD3+ thymocyte development in mice expressing the *lck*F505A20,23 transgene. Mice from the *lck*-CD4 727 line (41) were crossed to those from the *lck*F505A20,23 7120 line, and thymocyte CD3 histograms from progeny of a single cross are presented. Each animal's genotype is given (*top*) and the percentages of cells expressing CD3 at high and low densities are indicated.

Figure 9. Expression of p59*hck* in thymocyte extracts from *hck*F501 transgenic mice. Cell extracts were prepared from normal thymocytes (*NLC*), from *hck*F501-expressing thymocytes (*HYF 2173*), or from NIH 3T3 cells transfected with an *hck*F501 cDNA under transcriptional control of the metallothioein I promoter (MT 2.1) (53). 40 μg of total protein was resolved on 12% SDS-PAGE, transferred to nitrocellulose, and detected with an *hck*-specific antiserum. (→) Position of p59*hck*. There is also a slightly smaller unidentified crossreacting band in all lanes.

using an *lck* transgene that encodes a catalytically inactive form of p56*lck*. As the level of this protein increases, thymocyte production becomes progressively attenuated (28). Thus, the catalytically inactive form of p56*lck* acts as a dominant-negative inhibitor of p56*lck* function. Intriguingly, the thymocytes from animals bearing high levels of catalytically in-
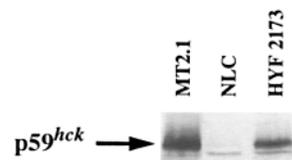
active p56*lck* support normal levels of Vβ gene rearrangement (28). These observations have prompted us to suggest that p56*lck* normally promotes thymocyte maturation by sensing the correct synthesis of a TCR β chain, and thereafter by directing that further rearrangements at the β locus cease and that cell replication proceed (28, 30). When p56*lck* is overexpressed in an active form, thymocytes are generated that appear to have received these signals inappropriately. In these cells, Vβ rearrangement is attenuated before satisfactory assembly of a functionally rearranged TCR β gene is complete. Although the specific targets of p56*lck* action remain undefined, it is apparent that the *lck*-encoded kinase acts relatively uniquely to deliver this signal, and that the signaling process requires protein tryosine kinase activity, since catalytically inactive p56*lck* does not promote the same effects (28).

Nature of the Signaling Structure that Activates p56^lck. Although the general function of *src*-family kinases remains mysterious, it is believed that these molecules assist in relaying signals from the cell surface to the cell interior. This conclusion derives in large part from the study of p56*lck* and p59*fyn* in lymphocytes. The hematopoietic isoform of p59*fyn* (p59*fynT*)

Table 2. *Summary of Results Obtained from hckF501 Transgenic Mice*

Line	Number	Expression	Transgene mRNA	p59*hck*	Tumor Formation	CD3+ (Thymus)	CD3+ (Periphery)
2169	1	23.5	+	ND	−	98	86
1637	1	ND	+	+	−	100	93
1629	1	ND	+	+	−	119	107
2186	1	ND	ND	ND	−	99	105
2189	1	ND	ND	ND	−	94	105
2173	1	ND	ND	+	−	96	113
2167	1	2.3	+	ND	−	ND	ND
13853	1	ND	ND	+	−	95	134
2171	3	ND	+	ND	−	107	ND
2195	3	ND	ND	+	−	100	ND

Expression values are reported as pg of transgene mRNA/μg of total RNA. Specific detection of transgene mRNA or p59*hck* when not quantitated is also noted. The number of CD3+ thymocytes and peripheral T cells is given as percentages of normal littermate control values. Tumor formation was judged by the same criteria as in Table 1.

interacts physically and functionally with the CD3 complex on T lymphocytes (33, 44–46), and loss of p59^{fyn} blocks antigen-induced signal transduction to a large extent, especially in mature thymocytes (45, 46). In contrast, p56^{lck} appears to interact with at least three, and perhaps more receptor structures. A unique motif in its NH$_2$-terminal domain confers upon it the ability to interact with the CD4 and CD8 coreceptors (17, 18), while its kinase domain can associate physically and functionally with the IL-2 receptor β chain (47). A recent report also documents the presence of p56^{lck} in immunoprecipitates formed using antisera to a variety of T cell surface proteins linked via a phosphatidylinositol-glycan moiety to the cell membrane (48).

In principle, any or all of these receptor structures might stimulate p56^{lck} to suppress thymocyte development. However, our data make plain that these signals do not arise from CD4 or CD8. In fact, augmented expression of CD4 appears to sequester p56^{lck} and prevent it from blocking Vβ gene rearrangement (Fig. 7). Thus animals of the 2954 lckF505 line, expressing approximately 12 pg/μg of transgene-derived transcript, behave more like those of the 3073 line, expressing 6 pg/μg of transgene mRNA (29), when a 15-fold excess of CD4 is present (Fig. 7 and data not shown). The inability of excess CD4 to rescue CD3 expression in thymocytes bearing an lckF505A20,23 transgene demonstrates the importance of the p56^{lck} cysteine motif in permitting CD4 to ameliorate the untoward effects of kinase overexpression. We have previously noted that expression of augmented levels of CD4 appeared to sequester endogenous p56^{lck} and block signaling in an antigen-specific thymocyte selection system, and that this ability depended upon the presence of the cytoplasmic tail of CD4 which contains a p56^{lck}-binding motif (41, 49). Similarly, Haughn et al. have proposed that sequestration of p56^{lck} by CD4 can explain the nonresponsiveness induced in a CD4$^-$ OVA-specific T cell line after expression of retrovirally encoded wild-type CD4 protein (50). Thus although CD4-mediated signaling in antigen-specific T cell lines may require interaction with p56^{lck} (19), potentiation of p56^{lck}-mediated responses is not dependent solely on CD4.

We can similarly conclude that the IL-2 receptor, though capable of binding p56^{lck}, probably does not activate its ability to arrest thymocyte development. First, p59^{fyn} can be shown to bind to the IL-2 receptor β chain and to be activated by IL-2 (51), and yet overexpression of p59^{fyn} does not

influence early thymocyte development (33). Second, mice lacking IL-2 as a result of a targeted gene disruption display no abnormalities in thymocyte maturation. In light of these observations, and since p56^{lck} expression is both required for normal thymocyte development, and incompatible with normal thymocyte development if it exceeds routine levels to any great extent, we conclude that p56^{lck} must ordinarily act through interactions with a receptor structure that is neither CD4, CD8, nor the IL-2 receptor β chain. In this context, it is important to note that p56^{lck} appears to promote signaling from the TCR itself in both a murine system, where overexpression of p56^{lck} improves antigen-specific responses in the complete absence of coreceptor expression (23), and in a human T cell tumor line, where loss of p56^{lck} expression blocks TCR signaling (24). These observations are compatible with the view that p56^{lck} functionally couples directly to the TCR–CD3 complex in mature T cells and thus could also do so during thymocyte development to sense the correct assembly of some portion of the TCR complex that depends upon β chain expression, but does not require either CD4 or CD8.

Selective Functions of src-Family Protein Tyrosine Kinases. The src-family protein tyrosine kinases are expressed in specialized forms in hematopoietic cells (2, 52). Indeed, 5/8 of these kinases (blk, fgr, hck, lck, and lyn) are expressed only in blood cells, and the fyn gene encodes two distinct proteins, one of which is hematopoietic cell specific (52). The effects of p56^{lck} on thymocyte development illustrate an emerging general principle: these nonreceptor kinases, even when expressed simultaneously in single cells, perform highly specialized signaling functions. Thus loss of fyn gene expression yields thymocytes that mature satisfactorily but fail to transmit normal TCR-derived activation signals (45, 46). In contrast, loss of lck gene expression blocks normal thymocyte development almost completely (27). Our studies of the effects of p56^{lck} overexpression add a further dimension to this analysis. Other src-family kinases, though similar in overall configuration and in their ability to phosphorylate substrates in vitro, simply cannot deliver a signal that suppresses β chain gene rearrangement. Definition of the structural features of p56^{lck} that endow it with its functional attributes should provide clues for the identification of molecules, both receptors and effectors, that regulate the development of T lymphocytes.

Address correspondence to Dr. Roger M. Perlmutter, Howard Hughes Medical Institute SL-15, University of Washington, Seattle, WA 98195. Kristin M. Abraham is currently at Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201-1559.

References

1. Marth, J.D., R. Peet, E.G. Krebs, and R.M. Perlmutter. 1985. A lymphocyte-specific protein-tyrosine kinase is rearranged and overexpressed in the murine T cell lymphoma LSTRA. *Cell.* 43:393.

2. Perlmutter, R.M., J.D. Marth, S.F. Ziegler, A.M. Garvin, S. Pawar, M.P. Cooke, and K.M. Abraham. 1988. Specialized protein tyrosine kinase proto-oncogenes in hematopoietic cells. *Biochim. Biophys. Acta.* 948:245.

3. Cooper, J.A. 1989. The *src* family of protein tyrosine kinases. *In* Peptides and Protein Phosphorylation. B. Kemp and P.F. Alewood, editors. CRC Press, Boca Raton. 85–113.

4. Voronova, A.F., and B.M. Sefton. 1986. Expression of a new tyrosine protein kinase is stimulated by retrovirus promoter insertion. *Nature (Lond.).* 319:682.

5. Reynolds, P.J., J. Lesley, J. Trotter, R. Schulte, R. Hyman, and B.M. Sefton. 1990. Changes in the relative abundance of type I and type II *lck* mRNA transcripts suggest differential promoter usage during T-cell development. *Mol. Cell. Biol.* 10:4266.

6. Bolen, J.B., P.A. Thompson, E. Eiseman, and I.D. Horak. 1991. Expression and interactions of the *src* family of tyrosine protein kinases in T lymphocytes. *Adv. Cancer Res.* 57:103.

7. Wildin, R.S., A.M. Garvin, S. Pawar, D.B. Lewis, K.M. Abraham, K.A. Forbush, S.F. Ziegler, J.M. Allen, and R.M. Perlmutter. 1991. Developmental regulation of *lck* gene expression in T lymphocytes. *J. Exp. Med.* 173:383.

8. Marth, J.D., J.A. Cooper, C.S. King, S.F. Ziegler, D.A. Tinker, R.W. Overall, E.G. Krebs, and R.M. Perlmutter. 1988. Neoplastic transformation induced by an activated lymphocyte-specific protein tyrosine kinase (pp56^{lck}). *Mol. Cell. Biol.* 8:540.

9. Amrein, K.E., and B.M. Sefton. 1988. Mutation of a site of tyrosine phosphorylation in lymphocyte-specific tyrosine protein kinase, p56^{lck}, reveals its oncogenic potential in fibroblasts. *Proc. Natl. Acad. Sci. USA.* 85:4247.

10. Abraham, K.M., S.D. Levin, J.D. Marth, K.A. Forbush, and R.M. Perlmutter. 1991. Thymic tumorigenesis induced by overexpression of p56^{lck}. *Proc. Natl. Acad. Sci. USA.* 88:3977.

11. Barber, E.K., J.D. Dasgupta, S.F. Schlossman, J.M. Trevillyan, and C.E. Rudd. 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase p56^{lck} that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. USA.* 86:3277.

12. Rudd, C.E., J.N. Trevillyan, L.L. Wong, J.D. Dasgupta, and S.F. Schlossman. 1988. The CD4 receptor is complexed to a T-cell specific tyrosine kinase, (pp58) in detergent lysates from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190.

13. Veillette, A., M.A. Bookman, E.M. Horak, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine protein kinase p56^{lck}. *Cell.* 55:301.

14. Veillette, A., M.A. Bookman, E.M. Horak, L.E. Samelson, and J.B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck} *Nature (Lond.).* 338:257.

15. Luo, K.X., and B.M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the *lck* tyrosine kinase at the autophosphorylation site. *Mol. Cell. Biol.* 10:5305.

16. Veillette, A., J.C. Zuniga-Pflucker, J.B. Bolen, and A.M. Kruisbeek. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J. Exp. Med.* 170:1671.

17. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Littman. 1990. Interaction of the unique N-terminal region of the tyrosine kinase p56^{lck} with the cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60:755.

18. Shaw, A.S., J. Chalupny, J.A. Whitney, C. Hammond, K.E. Amrein, P. Kavathas, B.M. Sefton, and J.K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56^{lck} tyrosine kinase. *Mol. Cell. Biol.* 10:1853.

19. Glaichenhaus, N., N. Shastri, D.R. Littman, and J.M. Turner. 1991. Requirement for association of p56^{lck} with CD4 in antigen-specific signal transduction in T cells. *Cell.* 64:511.

20. Perlmutter, R.M. 1989. T cell signaling. *Science (Wash. DC).* 245:344.

21. Biondi, A., C. Paganin, V. Rossi, S. Benvestito, R.M. Perlmutter, A. Mantovani, and P. Allavena. 1991. Expression of lineage-restricted protein tyrosine kinase genes in human natural killer cells. *Eur. J. Immunol.* 21:843.

22. Einspahr, K.J., R.T. Abraham, C.J. Dick, and P.J. Leibson. 1990. Protein tyrosine phosphorylation and p56^{lck} modification in IL-2 or phorbol ester–activated human natural killer cells. *J. Immunol.* 145:1490.

23. Abraham, N., M.C. Miceli, J.R. Parnes, and A. Veillette. 1991. Enhancement of T cell responsiveness by the lymphokine-specific tyrosine protein kinase p56^{lck}. *Nature (Lond.).* 350:62.

24. Straus, D.B., and A. Weiss. 1992. Genetic evidence for the involvement of the *lck* tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell.* 70:585.

25. Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmutter, and T. Tanaguchi. 1991. Interaction of the IL-2 receptor with the *src* family kinase p56^{lck}: identification of novel intermolecular association. *Science (Wash. DC).* 252:1523.

26. Horak, I.D., R.E. Gress, P.J. Lucas, E.M. Horak, T.A. Waldmann, and J.B. Bolen. 1991. T-lymphocyte interleukin 2–dependent tyrosine protein kinase signal transduction involves the activation of p56^{lck}. *Proc. Natl. Acad. Sci. USA.* 88:1996.

27. Molina, T.J., K. Kishihara, D.P. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C.J. Paige, K.U. Hartmann, A. Veillette, et al. 1992. Profound block in thymocyte development in mice lacking p56^{lck}. *Nature (Lond.).* 357:161.

28. Levin, S.D., S.J. Anderson, K.A. Forbush, and R.M. Perlmutter. 1993. A dominant-negative transgene defines a role for p56^{lck} in thymopoiesis. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1671.

29. Abraham, K.M., S.D. Levin, J.D. Marth, K.A. Forbush, and R.M. Perlmutter. 1991. Delayed thymocyte development induced by augmented expression of p56^{lck}. *J. Exp. Med.* 173:1421.

30. Anderson, S.J., K.M. Abraham, T. Nakayama, A. Singer, and R.M. Perlmutter. 1992. Inhibition of T-cell receptor β-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56^{lck}. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4877.

31. Kunkel, T.A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* 82:488.

32. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.

33. Cooke, M.P., K.M. Abraham, K.A. Forbush, and R.M. Perlmutter. 1991. Regulation of T cell receptor signalling by a *src* family protein-tyrosine kinase (p59^{fyn}). *Cell.* 65:281.

34. Chaffin, K.E., C.R. Beals, T.M. Wilkie, K.A. Forbush, M.I. Simon, and R.M. Perlmutter. 1990. Dissection of thymocyte signaling pathways by *in vivo* expression of pertussis toxin ADP-ribosyltransferase. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3821.

35. Ziegler, S.F., S.D. Levin, and R.M. Perlmutter. 1989. Transformation of NIH 3T3 fibroblasts by an activated form of p59^{hck} *Mol. Cell. Biol.* 9:2724.

36. Havran, W.L., M. Poenie, J. Kimura, R. Tsien, A. Weiss, and J.P. Allison. 1987. Expression and function of the CD3 antigen receptor on murine CD4$^+$CD8$^+$ thymocytes. *Nature (Lond.).* 330:170.

37. Leo, O., M. Foo, D.H. Sacha, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84:1374.

38. Louie, R.R., C.S. King, A. MacAuley, J.D. Marth, R.M. Perlmutter, W. Eckhart, and J.A. Cooper. 1988. p56^{lck} protein-tyrosine kinase is cytoskeletal and does not bind to polyomavirus middle T antigen. *J. Virol.* 62:4673.

39. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.

40. Allen, J.M., K.A. Forbush, and R.M. Perlmutter. 1992. Functional dissection of the *lck* proximal promoter. *Mol. Cell. Biol.* 12:2758.

41. Teh, H.-S., A.M. Garvin, K.A. Forbush, D.A. Carlow, C.B. Davis, D.R. Littman, and R.M. Perlmutter. 1991. Participation of CD4 coreceptor molecules in T-cell repertoire selection. *Nature (Lond.).* 349:241.

42. Perlmutter, R.M., S.D. Levin, M.W. Appleby, S.J. Anderson, and J. Alberola-Ila. 1993. Regulation of lymphocyte function by protein phosphorylation. *Annu. Rev. Immunol.* 11:451.

43. Ziegler, S.F., J.D. Marth, D.B. Lewis, and R.M. Perlmutter. 1987. Novel protein-tyrosine kinase gene (*hck*) preferentially expressed in cells of hematopoietic origin. *Mol. Cell. Biol.* 9:2724.

44. Timson-Gauen, L.K., A.N. Kong, L.E. Samelson, and A.S. Shaw. 1992. p59^{fyn} tyrosine kinase associates with multiple T cell receptor subunits through its unique amino-terminal domain. *Mol. Cell. Biol.* 12:5438.

45. Appleby, M.W., J.A. Gross, M.P. Cooke, S.D. Levin, X. Qian, and R.M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. *Cell.* 70:751.

46. Stein, P.L., H.-M. Lee, S. Rich, and P. Soriano. 1992. pp59^{fyn} mutant mice display differential signalling in thymocytes and peripheral T cells. *Cell.* 70:741.

47. Marth, J.D., D.B. Lewis, M.P. Cooke, E.D. Mellins, M.E. Gearn, L.E. Samelson, C.B. Wilson, A.D. Miller, and R.M. Perlmutter. 1989. Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56^{lck}). *J. Immunol.* 142:2430.

48. Stefanova, I., V. Horejsi, I.J. Ansotevi, W. Knapp, and H. Stockinger. 1991. GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science (Wash. DC).* 254:1016.

49. van Oers, N.S.C., A.M. Garvin, C.B. Davis, K.A. Forbush, D.A. Carlow, D.R. Littman, R.M. Perlmutter, and H.S. Teh. 1992. Disruption of CD8-dependent negative and positive selection of thymocytes is correlated with a decreased association between CD8 and the protein tyrosine kinase, p56^{lck}. *Eur. J. Immunol.* 22:735.

50. Haughn, L., S. Gratton, L. Caron, R.P. Sekaly, A. Veillette, and M. Julius. 1992. Association of tyrosine kinase p56^{lck} with CD4 inhibits the induction of growth through the alpha-beta T-cell receptor. *Nature (Lond.).* 358:328.

51. Shibuya, H., M. Yoneyama, J. Ninomiya-Tsuji, K. Matsumoto, and T. Taniguchi. 1992. IL-2 and EGF receptors stimulate the hematopoietic cell cycle via different signaling pathways: demonstration of a novel role for c-myc. *Cell.* 70:57.

52. Cooke, M.P., and R.M. Perlmutter. 1989. Expression of a novel form of the *fyn* proto-oncogene in hematopoietic cells. *New Biol.* 1:66.

53. Ziegler, S.F., C.B. Wilson, and R.M. Perlmutter. 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene (*hck*) after macrophage activation. *J. Exp. Med.* 168:1801.