

Control of Lymphopoiesis by p50^{csk}, a Regulatory Protein Tyrosine Kinase

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Summary

The *csk* gene encodes a nonreceptor protein tyrosine kinase that acts in part by regulating the activity of *src*-family protein tyrosine kinases. Since the *src*-family kinases p56^{lck} and p59^{lyn} play pivotal roles during lymphocyte development, it seemed plausible that p50^{csk} might contribute to these regulatory circuits. Using a gene targeting approach, mouse embryonic stem cell lines lacking functional *csk* genes were generated. These *csk*^{null} embryonic stem cells proved capable of contributing to many adult tissues, notably heart and brain. However, although *csk*^{null} progenitors colonized the developing thymus, T and B cell differentiation were both blocked at very early stages. This represented a relatively selective interdiction of lymphocyte maturation, since *csk*^{null} hematopoietic progenitors supported the development of normal-appearing MAC-1⁺ blood leukocytes, and the successful maturation of granulocyte/macrophage-colony-forming units from fetal liver progenitors. We conclude that p50^{csk} regulates normal lymphocyte differentiation, but that it almost certainly does so by acting on targets other than p56^{lck} and p59^{lyn}.

S*rc*-family protein-tyrosine kinases (PTK)¹ play crucial roles in regulating the proliferation and differentiation of multiple cell types. In part, this reflects their participation in the conveyance of signals initiated by the engagement of cell surface receptors with external ligands. The *src*-family of nonreceptor PTK genes consists of 10 known members, *c-src*, *c-yes*, *c-fgr*, *fyn*, *lyn*, *lck*, *hck*, *blk*, *yrk*, and *rak* (reviewed in references 1–3). Many of the family members are expressed in specific hematopoietic cell lineages and participate in regulating the development of these cells (reviewed in reference 4). For example, p56^{lck}, expressed early during thymocyte maturation, delivers signals that permit the maturation of CD4⁺8⁺ cells from CD4⁻8⁻ progenitors (5), whereas p59^{lyn} regulates activation responses in more mature T-lineage cells (6, 7). In all *src*-family kinases studied to date, regulation of catalytic activity is achieved in part through phosphorylation of a conserved COOH terminal tyrosine, which typically results in a 10- to 20-fold decrease in catalysis (8, 9). Some evidence suggests that phosphorylation at the COOH-

terminal tyrosine promotes an intramolecular or intermolecular interaction with the SH2 domain of the enzyme, thereby excluding other substrates from the active site (10).

Perhaps not surprisingly, phosphorylation of the COOH-terminal regulatory tyrosine of the *src*-family kinases appears to require the activity of an additional set of enzymes, of which the best studied is p50^{csk}. Structurally related to the *src*-family PTKs, but itself lacking the regulatory tyrosine phosphorylation site, p50^{csk} catalyzes phosphate transfer specifically to the COOH-terminal tyrosines of p59^{lyn}, p53/56^{lyn} (11), p60^{c-src} (12), and p56^{lck} (13) in vitro, and almost certainly subserves a similar function in vivo. For example, augmented expression of p50^{csk} suppresses transformation in *c-src/v-crk* transfected fibroblasts (14). Moreover, in *csk*-deficient fetal mice (generated by targeted gene disruption in embryonic stem cells) the activities of p60^{c-src} and p59^{lyn} are substantially increased. These mice fail to develop beyond embryonic day 9.5–10.5, and manifest severe defects in neurulation (15, 16). Whereas these observations do not exclude the possibility that p50^{csk} may act on other, unrelated targets (e.g., CD45; (17), they nevertheless implicate this kinase as a pivotal regulator of signal transduction via *src*-family kinases.

Immunoreactive p50^{csk} can be detected at low levels in al-

¹ Abbreviations used in this paper: ES, embryonic stem; PTK, protein tyrosine kinase; RAG-2, recombination-activating gene 2.

most all adult mouse tissues; however, it is predominantly expressed in adult thymus and spleen. This restricted expression pattern suggests that p50^{csk} may participate in the control of lymphocyte activation and/or development (12). Indeed, introduction of *csk* expression constructs into a T cell hybridoma both inhibited TCR signaling and abrogated the ability of p56^{lck} and p59^{lyn} to enhance the signaling process (18).

Previous studies clearly document the importance of p56^{lck} and p59^{lyn} catalytic function in the development of normal T cells. If p50^{csk} ordinarily regulates the activity of these kinases, inhibition of p50^{csk} function should yield animals with predictable phenotypes: the thymocytes from these mice should behave as if p56^{lck} and p59^{lyn} were inappropriately activated. Unfortunately, the early demise of *csk*^{null} fetuses prevented direct investigation of this hypothesis.

To evaluate further the *in vivo* role of p50^{csk}, we have generated chimeric mice by injection of normal and RAG (recombination activating gene)-2^{null} blastocysts with embryonic stem (ES) cells bearing a homozygous disruption of *csk*. In this system, the developmental potential of the *csk*^{null} cells was directly assessed using both semi-quantitative analysis of chimerism at the level of genomic DNA, and flow cytometric analysis of ES cell-derived populations. Remarkably, despite the profound block in development seen in *csk*^{null} embryos, ES cells bearing a homozygous disruption of the *csk* gene contributed significantly to numerous mature tissues, including the brain and the heart. These cells could not, however, support lymphopoiesis beyond the very earliest stages in either the T or B cell lineage. In a formal sense, these results demonstrate that disruption of the *csk* gene yields a cell-autonomous defect. Interestingly, *csk*^{null} ES cells proved perfectly capable of supporting myeloid cell development. These observations demonstrate that p50^{csk} acts to regulate differentiative events beginning at the time of lymphoid specification.

Materials and Methods

Generation of *csk*^{null} ES Cells. Growth of ES cells and targeted disruption of the first *csk* allele were performed as described in (6) using the gene disruption vector shown in Fig. 1 and described earlier (15). PCR was performed on genomic DNA made from 88 out of 301 G418-resistant clones. PCR products were produced using one primer from within the neomycin phosphotransferase gene (5'-TATCGCCTTCTTGACGAGTTCTTCTGAGGG) and a second primer from within the targeted gene outside the disruption vector (5'-GCCCTCAGTGGGCACCCCAAGCCCTATA). An additional 5' primer (5'-TCCCACCCGAGGGGAGTTTG-GTGGACTAT) was added to the reaction to amplify the endogenous *csk* gene. 11 recombinant clones were identified by the presence of a visible ethidium-stained band after PCR that was 200 bp smaller than that derived from the endogenous *csk* locus. The disruption event was confirmed by genomic blot analysis as described below. Gene disruption of the second allele was performed on four individual heterozygote ES clones by exposure to 4 mg/ml G418 for 8–11 d and expansion of surviving clones in 250 µg/ml G418 (19).

Generation of Chimeric Mice. ES cells were injected into C57BL/6 blastocysts which were then transferred into SW foster mothers as described (6). Adult and fetal progeny were analyzed to assess

chimerism by a number of criteria including coat color, Ly9 cell surface expression, and detection of the disruption construct by semi-quantitative PCR. A RAG^{null} mouse colony was established from breeders generously provided by Dr. Frederick W. Alt (Harvard Medical School, Boston, MA) (20). Blastocysts from these mice were injected using the same procedure as that employed from C57BL/6 blastocysts.

Immunoblot Blot Analysis. Whole cell lysates were prepared from ES cells (grown without feeder cells for three passages) as described (21). Samples containing 20 µg of protein were resolved by SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes and visualized according to previous protocols (15) with polyclonal antiserum specific for p50^{csk} (12). Immunoreactive proteins were detected using a donkey anti-rabbit peroxidase-conjugated second antibody, and visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL) followed in some cases by densitometry (model UA5; Isco Lincoln, NE).

Immunoprecipitation and Kinase Assays. Immunoprecipitation of p60^{c-src} proteins isolated from 20 µg of whole cell lysates was performed using mAb 327 as described (11). One fourth of the reaction was resolved on 10% SDS-PAGE and visualized using mAb 327 as described above. The remaining material was resuspended in kinase reaction buffer (20 mM Tris-Cl, pH 7.5, 10 mM MnCl₂, 1 mM Na₂VO₄, 0.1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin and leupeptin), and 2 µl per reaction of γ-[³²P]ATP [3,000 Ci/mmol] containing 2.5 µg of acid-treated enolase were added. Kinase reactions were performed for 1, 4, and 10 min at room temperature, stopped by addition of SDS-PAGE sample buffer and boiling for 5 min, and the products resolved on 10% SDS-PAGE. The incorporation of ³²P into protein bands was determined on an image analyzing system (model BAS2000; Molecular Dynamics, Sunnyvale, CA). Specific activity was calculated as incorporation of ³²P into either enolase or p60^{c-src} per unit of p60^{c-src} protein present.

Semi-quantitative PCR. Genomic DNA was prepared by homogenization of tissue culture cells or whole organs in 100 mM Tris-Cl, pH 7.5, 100 mM NaCl, 10 mM EDTA, 0.5% sarkosyl, and 100 µg/ml proteinase K at 55°C for 2 h or 37°C overnight. Extraction of the homogenates was performed with phenol, phenol/chloroform, and chloroform for at least 4 h each and DNA was precipitated with 2 vol of ethanol. PCR was performed on serial dilutions of DNA from *csk*^{null} ES cells (using the primers previously described to detect the disruption event) to ensure linearity of the reaction conditions. A standard curve was produced by mixing known quantities of DNA from *csk*^{null} cells with DNA from wild-type ES cells, to represent different levels of chimerism. PCR amplification of chimeric DNA was performed using the combination of primers that detects the disruption event and the endogenous *csk* locus on equal amounts of DNA representing different levels of chimerism. The same procedure was performed on several dilutions of test DNA from chimeric animals. The amplification products were resolved on 1% agarose gels, transferred to nitrocellulose, and probed with a ³²P-labeled oligonucleotide (5'-TGGAGG-CAGAGGTCACAGGTGCAGCTGATG). The incorporation of ³²P was quantitated using the BAS2000 image analyzing system or autoradiography.

Immunocytometry. Lymphocyte suspensions were prepared from whole organs and red blood cells lysed using ammonium chloride as previously described (7). Cells were stained using anti-CD4PE, anti-CD8FITC, anti-CD3FITC, anti-Ly9.1 Biotin, anti-Thy1.2FITC (PharMingen, San Diego, CA), and anti-B220PE (Caltag Labs, San Francisco, CA). Detection of biotinylated reagents was facili-

tated using PE conjugated streptavidin (Caltag Labs). Events were collected in list mode files on a FACScan® flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed using Reproman Software version 2.07 (Truefacts Software, Seattle, WA).

CFU-GM Assay and Analysis. Single cell suspensions of liver from fetal day 17 *csk*^{null} chimeric mice were prepared by digestion in PBS plus 20% FCS containing 0.75% collagenase D (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h followed by passage through a 22-gauge needle. The cells were plated at 5 × 10³ and 1 × 10⁴ cells/35 mm dish in 0.3% agar, over a layer containing 0.5% agar and growth factors (50 ng/ml stem cell factor, 2,300 U/ml IL-3, 20 ng/ml IL-6, 20 ng/ml IL-1, 4 U/ml erythropoietin, and 10 ng/ml GM-CSF). The cultures were allowed to develop for 8–10 d in 5% CO₂, 5% O₂, and 90% N₂. Colonies were harvested and analyzed using flow cytometry and the *csk*-specific PCR. Giemsa stained slides of cytospin cell preparations were also prepared. PCR reactions were performed by incubating the cells in PCR reaction buffer including 20 pmol oligonucleotide primers, 10 μg/ml proteinase K, 0.1% Triton X and 0.1% NP-40 at 56°C for 1 h followed by 4 min at 94°C. 0.5 μl of Taq polymerase was thereafter added to each reaction, and PCR was performed as described above.

Results

Production of ES Cells Lacking Both Alleles of the *csk* Gene. Targeted disruption of the *csk* gene was performed

by electroporation of ES cells with the construct shown in Fig. 1 A (15). Homologous recombination deletes most of the coding region and replaces it with the neomycin phosphotransferase expression cassette and the *Escherichia coli lacZ* gene. Positive/negative selection was achieved by flanking the construct with sequences encoding diphtheria toxin (22). After electroporation and G418 selection, 301 clones were expanded and 11 out of 88 clones tested by PCR were identified as having sustained a gene disruption event at the *csk* locus. These results were confirmed by genomic blot analysis (Fig. 1 B). All 11 clones contained a single integration of the neomycin phosphotransferase sequence (data not shown). ES cells which lacked both alleles of the *csk* gene were obtained after exposure of *csk* heterozygotes to 4 mg/ml G418 (19). Genomic blot analysis of DNA digested with HindIII and probed with an internal KpnI-EcoRI fragment demonstrated the appropriate distribution of wild-type (10 kb) and disrupted (6.8 kb) fragments in cells of the three different genotypes (Fig. 1 B).

To document the effect of homozygous disruption of the *csk* gene, we evaluated putative *csk*^{null} ES cells for the presence of immunoreactive p50^{csk}, and for alterations in the specific activity of p60^{src}. Fig. 2 A shows that the level of p50^{csk} protein in ES cells directly reflects *csk* gene dosage; *csk*^{+/-} cells express 50% less p50^{csk} protein compared with wild-type cells, while *csk*^{null} cells contain no immunoreac-

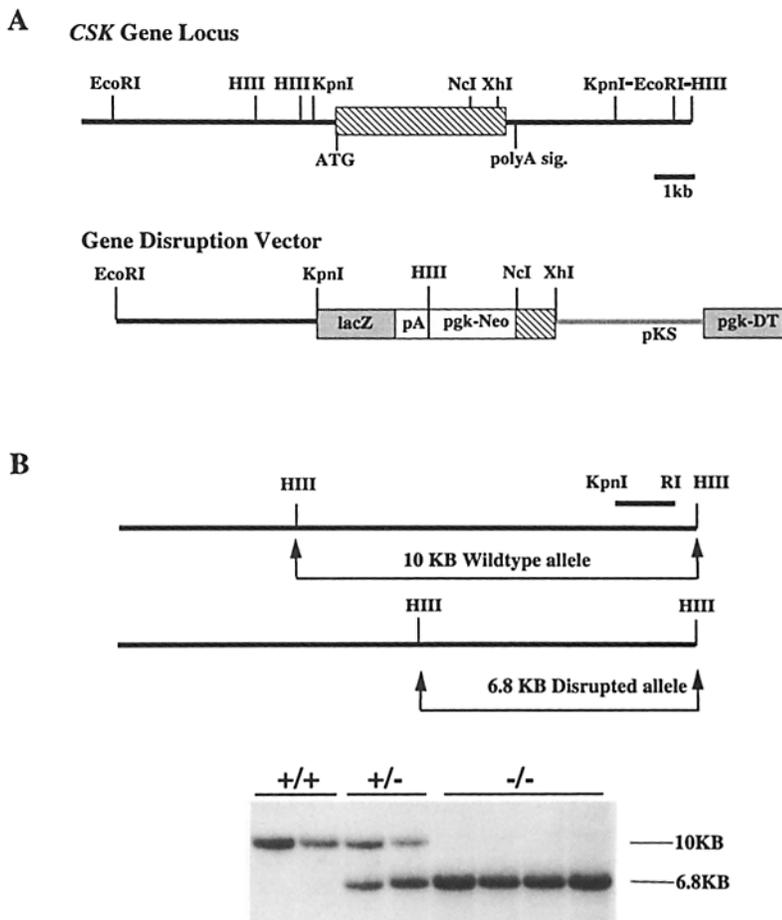


Figure 1. *csk* gene disruption strategy. (A) Partial restriction map of the *csk* locus showing the coding region as a hatched box. The *csk* gene disruption vector replaces the KpnI to NcoI fragment with a neomycin resistance expression cassette (*pgk-Neo*) and *lacZ* gene and contains the diphtheria toxin gene (*pgk-DT*) at the 3' end of the construct. (B) Southern blot analysis of genomic DNA prepared from parental ES cells (+/+), *csk* heterozygous ES cells (+/-), and *csk*^{null} ES cells (-/-) digested with HindIII was probed with a 1.5-kb KpnI to EcoRI *csk* gene fragment. The wild-type *csk* allele resolves at 10 kb and the disrupted allele migrates at 6.8 kb on 1% agarose gels.

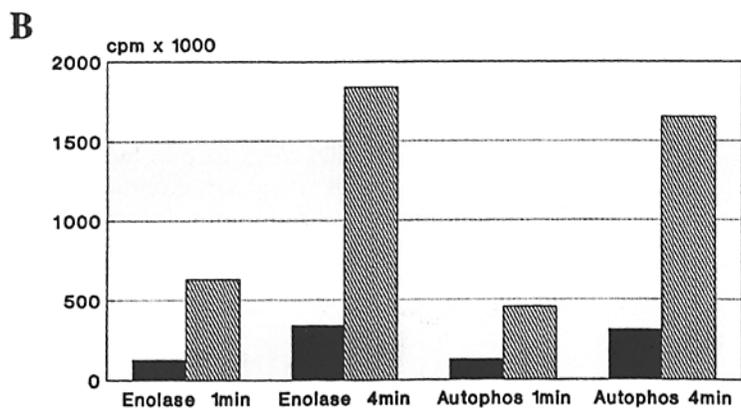
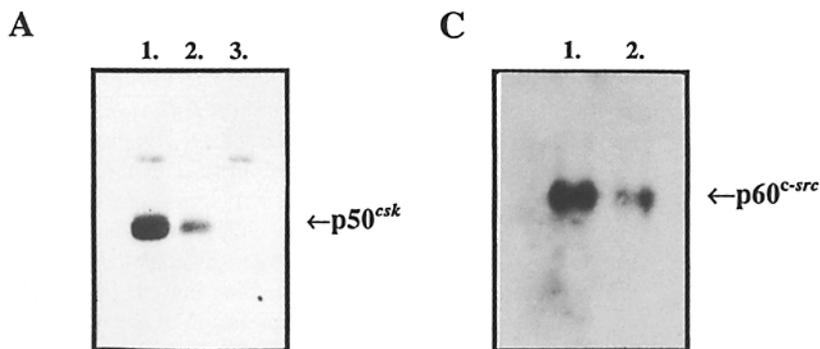
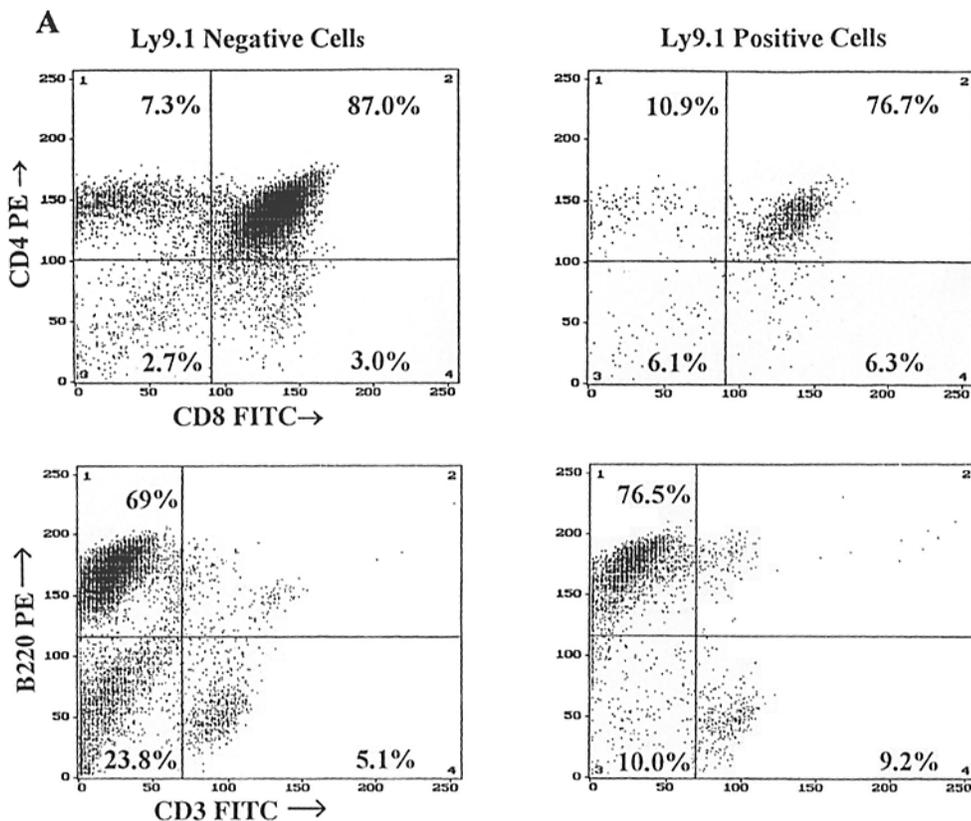


Figure 2. Absence of p50^{csk} and activation of p60^{c-src} in *csk*^{null} ES cells. (A) Immunoblot analysis reveals no p50^{csk} protein in whole cell lysates isolated from *csk*^{null} ES cells (lane 3) as compared with levels of p50^{csk} detected in *csk*^{+/-} ES cells (lane 2) and wild-type ES cells (lane 1). (B) The specific activity of p60^{c-src} was determined by measuring the kinase activity of p60^{c-src} immunoprecipitates isolated from wild-type ES cells (dark bars) and *csk*^{null} ES cells (hatched bars). Kinase reactions were performed for 1 and 4 min, measuring the ability of p60^{c-src} to incorporate γ -[³²P]ATP by autophosphorylation, or by phosphorylation of an exogenous enolase substrate. (C) The amount of p60^{c-src} was determined in each sample by immunoblot analysis from wild-type cells (lane 1) and *csk*^{null} cells (lane 2) to calculate the specific activity from each reaction.



tive p50^{csk}. Moreover, the specific activity of p60^{c-src} immunoprecipitated from *csk*^{null} ES cells increased at least sixfold as compared with the measured activity in wild-type ES cells when judged either by autophosphorylation, or by phosphorylation of an exogenous substrate (Fig. 2 B). There was a similar increase in the specific activity of p59^{fyn} in *csk*^{null} ES cells (data not shown). Interestingly, the amount of p60^{c-src} protein in *csk*^{null} ES cells was markedly less than in wild-type cells (Fig. 2 C), an effect previously observed in *csk*^{null} embryos (15). Thus by direct evaluation these *csk*^{null} ES cells do not express a functional *csk* gene product.

csk^{null} ES Clones Do Not Support Development of Adult Lymphocytes. To determine if p50^{csk} is required for the normal development of lymphoid cells, we produced chimeric mice using ES cells lacking both alleles of p50^{csk}. Three indepen-

dently-derived *csk*^{null} ES cell clones (301A.104, 189A.40, and 153A.50), themselves derived from unrelated *csk*^{+/-} ES cell clones (301A, 189A, and 153A) were injected into C57BL/6 blastocysts, which were then transferred into SW foster mothers. The ES cells, derived from the 129/Sv mouse strain, yield progeny lymphocytes bearing the Ly9.1 surface marker, whereas the C57BL/6 host-derived cells express only the Ly9.2 allele.

To document the efficacy of this approach, *csk* heterozygote clones 92A, 189A, and 153A were injected into C57BL/6 blastocysts. These animals exhibited substantial coat color chimerism, and between 10 and 65% of peripheral blood leukocytes expressed the Ly9.1 marker (data not shown). Thymocytes and splenocytes from these mice were analyzed by three-color flow cytometry to ensure that normal subpopulations

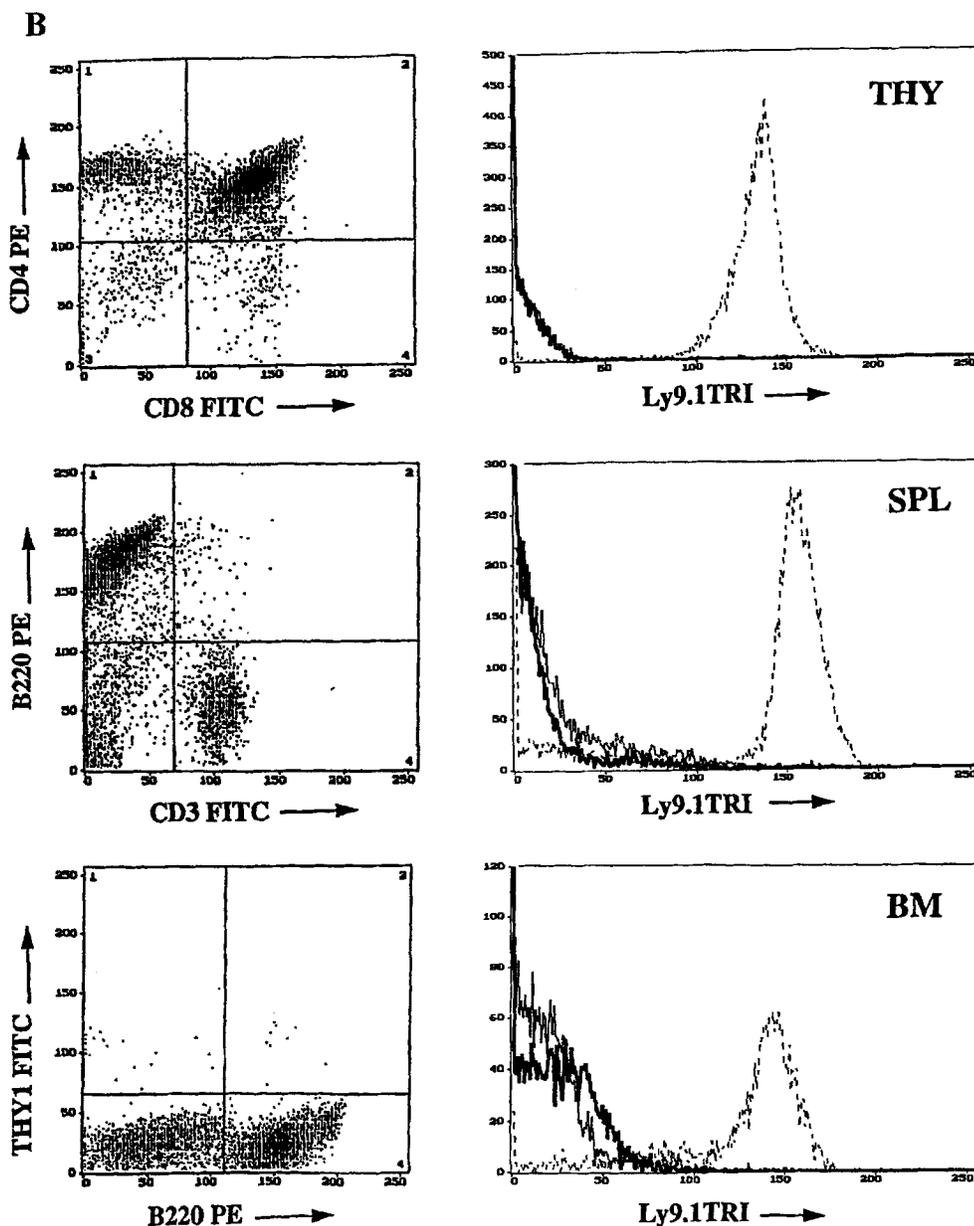


Figure 3. Three-color flow cytometric analysis of lymphocytes and lymphocyte progenitors from adult *csk*^{+/-} and *csk*^{null} chimeric mice. (A) Three-color flow cytometry was performed on thymocytes and splenocytes from 4-wk-old mice chimeric for *csk*^{+/-} ES cells using combinations of mAbs shown and Ly9.1 biotin/tricolor streptavidin (TriSA). Electronic gating was performed on Ly9.1 negative cells (left) and Ly9.1 positive cells (right) which were then analyzed for expression of CD4 and CD8 on thymocytes (top) and for expression of B220 and CD3 on splenocytes (bottom). (B) Three-color flow cytometry was performed on thymocytes, splenocytes, and bone marrow cells isolated from 4-wk-old *csk*^{null} chimeric mice using combinations of mAbs shown and Ly9.1 TriSA. Two-parameter histograms are plotted in the left column and histogram overlays of the same samples stained with Ly9.1 (dark line) and an isotype-matched control antibody (thin line) are in the right column. A separate sample was stained to demonstrate the expression of Ly9.1 on cells derived from a Ly9.1-positive age-matched strain (dotted line).

were represented in the *csk* heterozygote-derived lymphocytes. Both *csk*^{+/-} (Ly9.1⁺) and host (Ly9.1⁻) populations contained comparable thymocyte subsets as defined by expression of CD4 and CD8, and exhibited comparable development of mature T and B cells as defined by the cell surface markers B220 and CD3 (Fig. 3 A). In contrast, the *csk*^{null} ES cells, derived directly from the heterozygous clones for which assays are shown in Fig. 3 A, proved incapable of contributing to the adult lymphoid compartment. Three-color flow cytometry was performed on cells obtained from the thymuses, spleens, and bone marrow of 14 adult animals, all of which were chimeric as judged by coat color. No Ly9.1⁺ cells were detected in any of these tissues (Fig. 3 B). Despite the absence of mature lymphocytes derived from the *csk*^{null} ES cells, host precursors appeared to differentiate normally, indicating that the presence of the *csk*^{null} stem cells had little effect on the development of endogenous wild-type progenitors (see below). Moreover, since the defect in the *csk*^{null} progenitors could not be corrected even by the presence of an overwhelming number of wild-type cells in the chimeras, loss of *csk* expression can be said to yield a cell autonomous phenotype.

Contribution of *csk*^{null} ES Cells to Other Mouse Tissues. To assess the ability of *csk*^{null} ES cells to participate in the development of various organs of the whole animal, we developed a semi-quantitative PCR assay capable of detecting the disrupted *csk* allele in the presence of excess wild-type genomic DNA (see Materials and Methods). Evaluation of tissue homogenates from thymus, spleen, heart, brain, kidney, liver, and bone marrow from 10 *csk*^{null} chimeric animals revealed that *csk*^{null} ES cells can contribute to the overall body plan (Fig. 4, A and B), however the level of chimerism was typically quite low. Consistent skewing was observed in the representation of *csk*^{null}-derived genomic DNA, such that heart (8% chimerism) and brain (5% chimerism) invariably contained the largest representation of *csk*^{null} cells, followed by kidney and liver (about 1% chimerism), and thereafter by bone marrow, spleen, and thymus. Since this assay was performed using entire organs, we cannot identify precisely which cells in each tissue were derived from the *csk*^{null} ES cells. However the overall low level of ES cell contribution found in the adult animals demonstrates that the developmental potential of *csk*^{null} cells in many lineages is impaired relative to that of *csk* heterozygote ES clones, which produce significantly higher levels of chimerism ranging between 10 and 65% in all organs tested (data not shown).

The ability of the *csk*^{null} ES clones to produce viable chimeric animals also provides information about the overall developmental potential of ES cells with this mutation. We have compared the litter sizes of chimeric mice made from the injection of *csk*^{null} ES clones with those observed after injection of control cells, including wild-type ES cells, *csk*^{+/-} ES cell clones, and *fyn*^{null} ES cell clones (which support normal embryogenesis; 6). The *csk*^{null} chimeric blastocysts produced an average of 2.7 pups per pregnant female out of 20 pregnancies tested, whereas the average litter size derived from the injection of control cells was 5.4 pups per pregnant

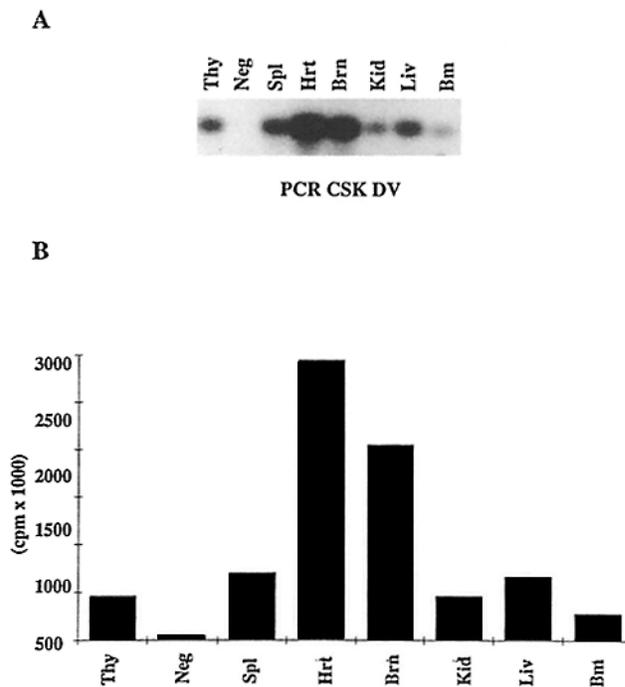


Figure 4. Detection of *csk*^{null} cell DNA by PCR in tissues of adult chimeric mice. Genomic DNA was isolated from homogenates of thymus, spleen, heart, brain, kidney, liver, and bone marrow from *csk*^{null} chimeric mice. (A) Semi-quantitative PCR yielded fragments that were resolved on 1% agarose, transferred to nitrocellulose, and probed with an internal γ -[³²P]ATP-labeled oligonucleotide. (B) The incorporation of ³²P was quantitated using a BAS2000 image analyzing system, and results were plotted for each tissue.

female examining 17 pregnancies ($p < 0.0005$ by Student's *t* test). This difference in litter size indicates, in accord with the early lethality observed in *csk*^{-/-} homozygote embryos (15, 16), that high levels of chimerism are not tolerated by the fetal mouse.

***csk*^{null} ES Clones Can Develop into Immature Thymocytes in Fetal Chimeric Mice.** Since *csk*^{null} ES cells appeared capable of contributing to many cell populations, at least at low levels, we asked whether *csk*^{null} lymphoid progenitors, though undetectable in adult chimeras, might exist in chimeras examined at earlier developmental time points. This proved to be the case. Fig. 5 presents the results obtained when three-color flow cytometric analysis was performed on thymocytes from a typical *csk*^{null} chimera examined at day 19 of gestation. In this animal, 5.8% of the thymocytes were derived from the *csk*^{null} ES cell line, as judged by Ly9.1 staining. Interestingly, the Ly9.1⁺ cells in these mice contained a larger proportion of less mature CD4⁻8⁻ (double negative) cells (12.2%) and CD8^{lo} cells (17.8%) as compared with what was found in wild-type (Ly9.1⁻) thymocytes (5.6 and 3.4%, respectively). Concomitantly, the representation of CD4⁺8⁺ (double positive) cells was dramatically reduced (39.4% compared to 83% for host double positive cells), and these cells appeared abnormal in that they expressed reduced levels of CD4. Indeed, a large subset of CD4^{dim} cells, which may represent an early

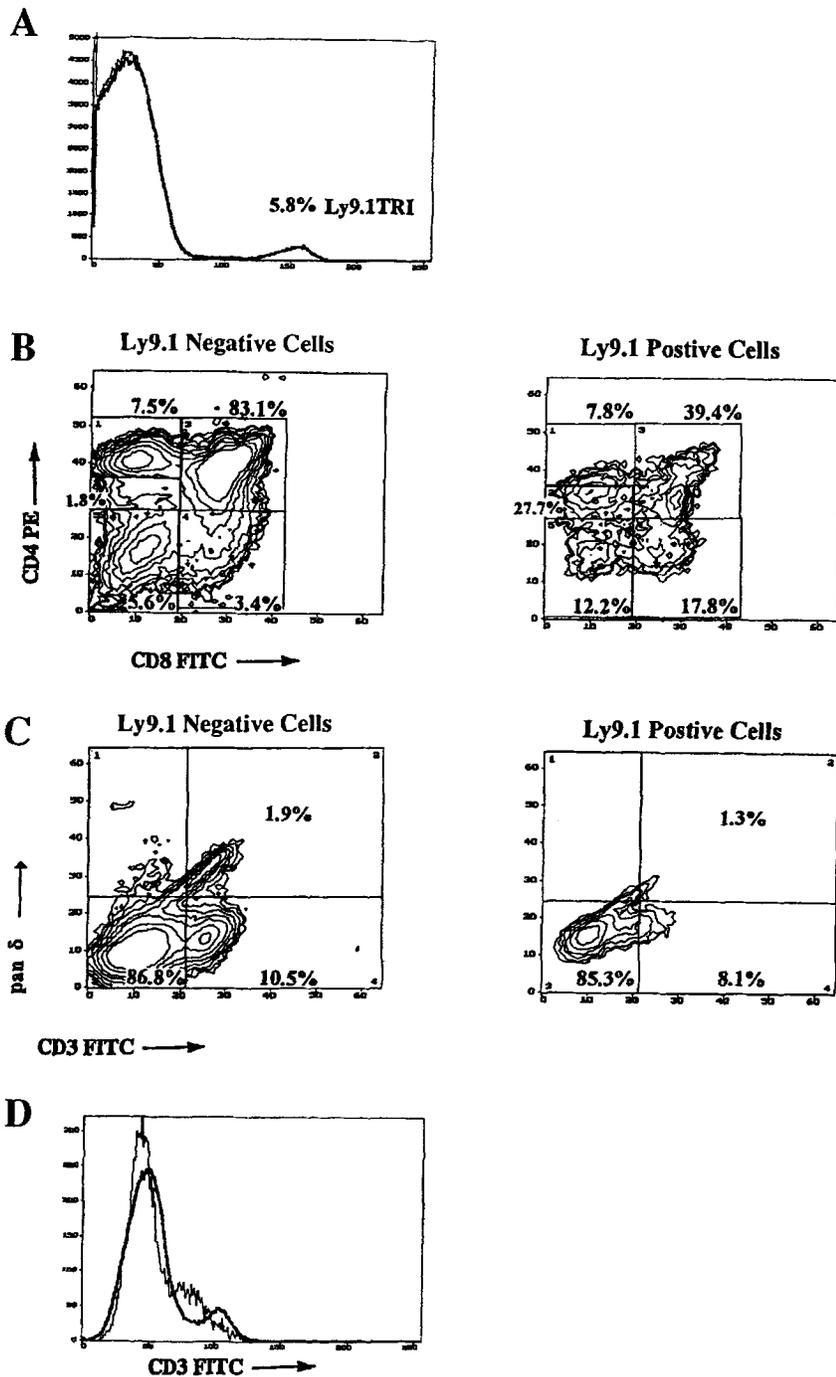


Figure 5. *csk*^{null} ES cells can develop into immature thymocytes in the fetal chimeric mouse. Three-color flow cytometry was performed on fetal day 19 thymocytes derived from *csk*^{null} chimeric mice. Cells were stained with combinations of anti-CD4PE and anti-CD8FITC, anti-TCR-δPE and anti-CD3 FITC, together with Ly9.1 biotin/TrISA. (A) Overlay of Ly9.1 staining (dark line) and control mAb (thin line) on fetal thymocytes. (B) CD4 and CD8 staining of the same cells electronically gated to include Ly9.1-negative cells (left) and Ly9.1-positive cells (right). (C) CD3 versus anti-TCR-δ staining of thymocytes gated on Ly9.1-negative and Ly9.1-positive cell populations. (D) Overlay of CD3 staining on Ly9.1-positive cells (thin line) and Ly9.1-negative cells (dark line) from same sample as in C.

thymocyte immigrant (23), was appreciable in the Ly9.1⁺ population. Very few mature cells, expressing CD3ε, were represented among the Ly9.1⁺ cells (Fig. 5 D). This inability of *csk*^{null} stem cells to yield phenotypically mature intrathymic progeny was confirmed in fetal day 17 chimeric mice (data not shown).

Reconstitution of RAG-2^{null} Blastocysts with *csk*^{null} ES Clones. Although the differentiative capability of *csk*^{null} T cell progenitors appeared to be severely compromised, it was possible that this phenotype resulted from inefficient competi-

tion with wild-type T-lineage cells in the chimeric thymus. To examine more directly the innate ability of *csk*^{null} intrathymic precursors to give rise to T cell progeny, we generated additional chimeras using RAG-2^{null} blastocysts as hosts. In these animals, thymopoiesis arrests at the CD4⁻8⁻, IL-2Rα⁺ stage (19, 24). Hence this environment should permit development of *csk*^{null} cells unhindered by host cell competition. Nevertheless, as in the chimeras generated using wild-type blastocysts, *csk*^{null} ES cells proved incapable of contributing to the development of adult lymphoid populations

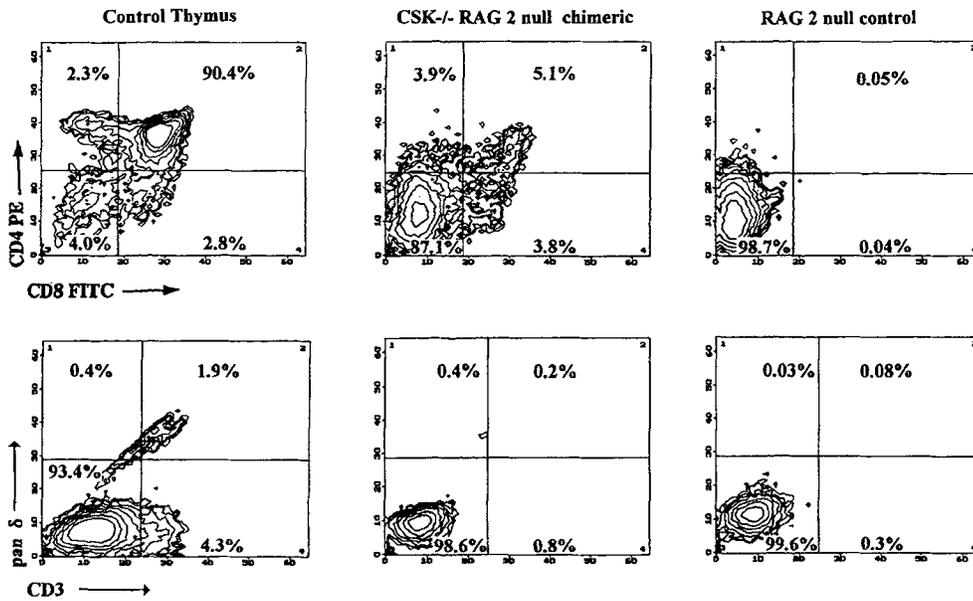


Figure 6. Reconstitution of RAG-2^{null} blastocysts with *csk*^{-/-} ES cells in the fetal thymus. Two-color flow cytometry was performed on fetal day 19 thymocytes obtained from control mice, chimeric mice made from RAG-2^{null} blastocysts reconstituted with *csk*^{-/-} ES cells, and RAG-2^{null} controls. Histograms of CD4 vs. CD8 and CD4 vs. anti-TCR- δ are shown.

(data not shown). Analysis of *csk*^{null}/RAG-2^{null} chimeric mice at fetal day 19 once again defined the limits of intrathymic differentiative capability for *csk*^{null} cells. Immature CD8^{lo} and CD4^{hi} CD8⁺ DP thymocytes, representing 3.8 and 5.1% of total thymocytes respectively (Fig. 6), were once again present, however CD3⁺ cells did not appear in appreciable numbers. Note in particular the absence of a significant CD3^{lo} population, that which ordinarily represents about half of all CD4^{hi} CD8⁺ cells (Fig. 6). Even γ/δ T cells, which share a very early progenitor with α/β T cells (25), fail to develop from

csk^{null} precursors (Fig. 6). Again, a relatively large number (3.9% of the total) of CD4^{lo} cells, not immediately related to any normally abundant thymocyte population, emerges in the *csk*^{null} chimeric thymuses. We conclude that progenitor cells lacking the *csk* gene cannot support a normal program of thymocyte maturation, even when permitted to develop in the relative absence of competitive influences.

Mature Myeloid Cells Develop From *csk*^{null} Precursors. The inability of *csk*^{null} progenitors to give rise to normal T or B lymphocytes (Fig. 3 B) suggested that *csk* function might

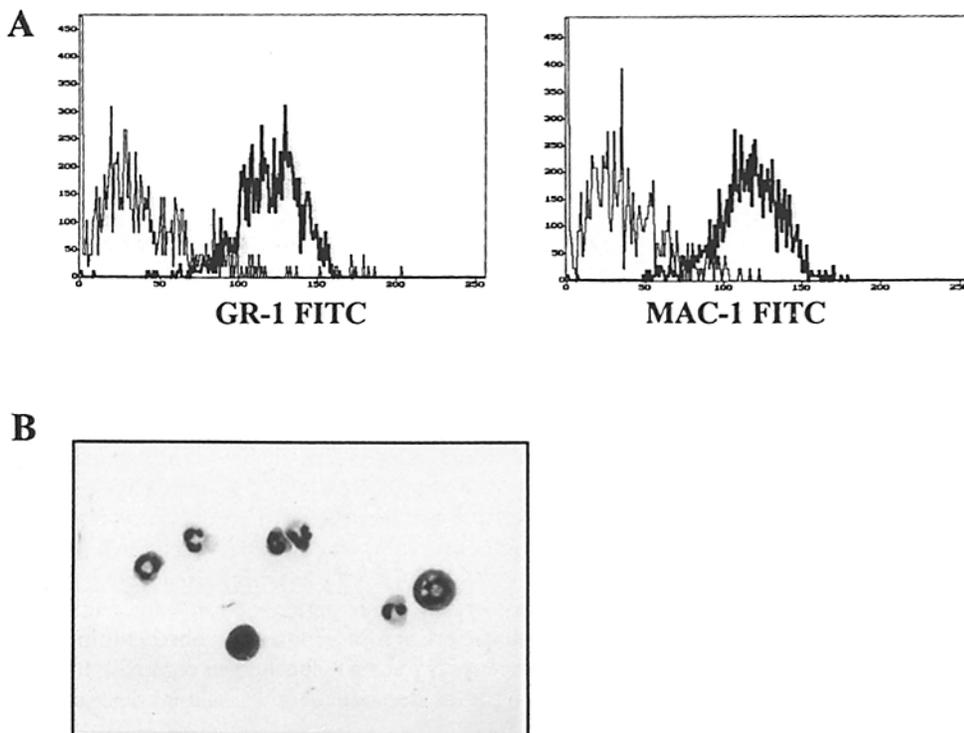


Figure 7. Development of granulocyte-myeloid lineage cells in CFU-GM assays. CFU-GM assays were performed on fetal liver cells obtained from fetal day 17 *csk*^{null} chimeric mice. (A) Analysis of a colony subsequently shown by PCR to contain only *csk*^{null} cells for expression of GR-1 or MAC-1 myeloid markers plotted as a single-parameter histogram. (Thin lines) Background staining with a control antibody. (B) Appearance of cells from a *csk*^{null} CFU-GM colony revealed by Giemsa staining and subsequent photomicroscopy. Mature granulocyte nuclei can be readily appreciated.

be required in a general way to permit hematopoietic cell differentiation. Nevertheless, detectable numbers of *csk*^{null} cells, representing either stromal cells or other leukocytes, appeared in the spleens of chimeric animals as judged by PCR amplification of DNA (Fig. 4). Moreover, as much as 4.6% of the GR-1⁺ Mac-1⁺ splenocytes were ES cell-derived (data not shown). To assess the ability of *csk*^{null} ES clones to develop within the myeloid linkage, single cell suspensions derived from livers of *csk*^{null} chimeric fetal mice were allowed to develop in a CFU-GM assay (26). PCR analysis of individual colonies permitted direct assessment of cellular provenance. In three separate experiments, all colonies were found to be clonal, and 25% were of *csk*^{null} origin. These *csk*^{null} myeloid colonies contained mature granulocytic and monocytic cells as judged by staining with anti-Mac-1 and anti-GR-1 antibodies (Fig. 7 A), and by histology (Fig. 7 B). We conclude that with respect to leukocyte development, the *csk*^{null} mutation leads to a relatively selective deficiency in lymphopoiesis.

Discussion

Considerable evidence supports the hypothesis that *src*-family PTKs play crucial roles in the development and activation of hematopoietic cells (reviewed in reference 4). For example, p60^{c-src} itself must be expressed to permit normal osteoclast development (27), whereas a deficiency of p56^{lck} leads to a very early block in thymopoiesis (28). The latter example provides an especially important paradigm for understanding the way in which signals from nonreceptor protein tyrosine kinases act to control development. Direct manipulation of the *lck* gene in mouse embryos has demonstrated that p56^{lck} serves as a component of a sensing mechanism which informs developing thymocytes when satisfactory expression of a TCR- β chain protein has occurred (5, 29). The details of this phenomenon were appreciated in part with the realization that provision of augmented p56^{lck} activity suppresses rearrangement of V β gene segments, promotes rearrangement of V α gene segments, and simultaneously drives the proliferative expansion and maturation of CD4⁺8⁺ cells (30). Activation of p56^{lck} can be stimulated through dephosphorylation of Tyr505, the site of tyrosine phosphorylation by p50^{csk}. Since Tyr505 is also the principle site of tyrosine phosphorylation of p56^{lck} in vivo (31, 32), there was reason to believe that p50^{csk} might serve as a component of the regulatory machinery that ordinarily permits activation of p56^{lck} in response to TCR β chain expression.

The p50^{csk} kinase is one of an emerging group of non-receptor protein tyrosine kinases with apparent specificity for the COOH-terminal regulatory tyrosines of *src*-family members. Among the *csk*-like kinase genes are murine *ctk* (33) which is the mouse homologue of human *MATK* (34), *HYL* (35), *lsk* (36), and the mouse *ntk* gene (37). While little is known about these recently discovered PTKs, there is reason to believe that each may be involved in regulating *src*-family kinase activity in distinct cell lineages. Thus the *HYL* gene is expressed primarily in myeloid cells, whereas the *MATK* kinase is expressed at high levels in megakaryocytes. In contrast, p50^{csk}, though expressed in fetal brain and at very low

levels in most adult tissues, is expressed at highest levels in adult thymocytes and splenocytes (11). Hence this protein is an attractive candidate for regulating p56^{lck}. In its simplest form, the hypothesis that p50^{csk} acts to regulate p56^{lck} signaling (perhaps to suppress delivery of the *lck*-derived signal until TCR- β chain synthesis is achieved) predicts that targeted disruption of the *csk* gene should mimic, in thymocytes, expression of an activated *lck* mutant transgene. We have tested this prediction directly, and find instead that p50^{csk} plays a much more fundamental role in regulating lymphocyte development.

Several important features of p50^{csk} function were illuminated by examining chimeric mice generated using *csk*^{null} ES cells. First, ES cells lacking p50^{csk} protein are indistinguishable from wild-type cells with respect to in vitro growth properties, even though the activity of p60^{c-src} in such cells is significantly elevated (Fig. 2). Thus, p50^{csk} does not participate in any unique way in the control of routine cell growth. This observation was, in effect, expected since *csk*^{-/-} embryos develop at least until day 9.5 of gestation (15, 16). At the same time, however, the ability of *csk*^{null} ES cells to contribute to histiogenesis in the mouse was severely impaired. Only a few percent of the cells in most tissues were derived from the *csk*^{null} ES cells rather than the host blastomeres.

One intriguing feature of the *csk*^{null} ES cells was the systematic variation in their ability to contribute to adult tissues. Thus the level of chimerism in the heart and brain, though much lower than that observed using *csk*^{+/+} ES cells, was nevertheless >20-fold better than that seen in the thymus or spleen (Fig. 4). Although we were unable to define precisely which cells in the heart and brain were ES cell derived, the differential impairment wrought by blocking *csk* expression argues that this PTK functions differentially to control the maturation of selected cell lineages.

This specificity of p50^{csk} effects can be seen most clearly in hematopoietic populations. Precursors derived from *csk*^{null} ES cells cannot support normal lymphopoiesis, but instead give rise only to very immature cell populations that never express normal antigen receptor complexes and that disappear shortly after birth. This represents a defect peculiar to the lymphoid lineage among white blood cells, since mature-appearing granulocytes and monocytes differentiate readily from *csk*^{null} hematopoietic progenitors and can be found among adult splenocytes. It is possible that the *HYL* gene product, or another related kinase, regulates myeloid cell development (much as p50^{csk} controls lymphopoiesis), permitting appropriate differentiation even in a *csk*^{null} background. Importantly, both T and B cell generation are effectively stymied in chimeras made using *csk*^{null} ES cells, implying either that p50^{csk} acts in a precursor cell common to both populations (the so-called lymphoid stem cell; 25) or that both T and B cell precursors independently traverse developmental checkpoints that require p50^{csk} activity.

Regardless of the site of action of p50^{csk} in lymphopoiesis, the characteristics of the defect observed in *csk*^{null} thymocytes permit important conclusions regarding its function. We initiated these studies with the expectation that p50^{csk} might participate in regulating p56^{lck} or p59^{lyn}. How-

ever inappropriate activation of p56^{lck} promotes, rather than suppresses, the development of CD4⁺8⁺ thymocytes, and activated p59^{lyn}, even when expressed at high levels in immature thymocytes, does not perturb T cell development (5, 21, and data not shown). Our data are most compatible with the view that p50^{csk} acts on targets other than the *src*-family kinases known to regulate T cell development. The recent observation that p50^{csk} can phosphorylate and activate the CD45 phosphotyrosine phosphatase supports this view, in the sense that there exist demonstrable substrates for p50^{csk} other than *src*-family kinases.

What can be said of the intrathymic process regulated by p50^{csk}? Clearly, the activity of this protein is not required to permit colonization of the fetal thymic anlage by hematopoietic stem cells, since the level of chimerism in early T-lineage precursors approaches the maximum that can be seen in any tissue. However a very substantial proportion of these cells, nearly 10% when wild-type blastocysts were used as hosts and an even larger fraction in RAG^{null} blastocyst reconstitutions, was phenotypically CD4^{lo}8⁻ (Figs. 5 and 6). Cells with this phenotype, while exceedingly rare among normal thymocytes, reportedly contain very early progenitors capable of giving rise to either T or B lymphocytes (23). It is therefore attractive to propose that *csk*^{null} progenitors successfully colonize the thymus as CD4^{lo} cells, but that differentiation thereafter proceeds very poorly, since the

representation of CD4⁻8⁻, CD4⁻8^{lo}, and CD4⁺8⁺ cells is far less than what would be expected given the abundance of the CD4^{lo}8⁻ precursors. It is worth noting that the absence of *csk*^{null}-derived lymphocytes in the adult mouse thymus, when significant numbers of immature thymocytes are present in fetal thymuses, means that *csk*^{null} stem cells, which in principle should continue to populate the thymus throughout life in small numbers, become progressively less able to compete effectively, even when matched against RAG^{null} progenitors. This observation emphasizes the pivotal role of this nonreceptor PTK in regulating thymopoiesis.

Lastly, we note that our observations do not necessarily undermine the assertion that p50^{csk} controls p56^{lck} activity and hence the maturation of CD4⁺8⁺ cells from CD4⁻8⁻ thymoblasts. Instead, since p50^{csk} clearly regulates an even earlier developmental transition, the involvement of p50^{csk} in modulating p56^{lck} activity could not be addressed. Detailed analysis of the stage-specific functions of p50^{csk} will require novel strategies to direct elimination of this protein, or inhibition of its activity, in selected thymocyte subpopulations (38). For the present, we conclude that expression of p50^{csk} is selectively required for the satisfactory maturation of early lymphoid progenitors. Elucidation of the mechanism whereby p50^{csk} entrains lymphopoiesis should also illuminate the role of this kinase, and its structural relatives, in controlling development in other cell lineages.

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