Control of Lymphopoiesis by p50ck, 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 p56ck and p59ftn play pivotal roles during lymphocyte development, it seemed plausible that p50ck might contribute to these regulatory circuits. Using a gene targeting approach, mouse embryonic stem cell lines lacking functional csk genes were generated. These csk−/− embryonic stem cells proved capable of contributing to many adult tissues, notably heart and brain. However, although csk−/− 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−/− 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 p50ck regulates normal lymphocyte differentiation, but that it almost certainly does so by acting on targets other than p56ck and p59ftn.

Src-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, yck, 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, p56ck, expressed early during thymocyte maturation, delivers signals that permit the maturation of CD4−8+ cells from CD4−8− progenitors (5), whereas p59ftn 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 p50ck. Structurally related to the src-family PTKs, but itself lacking the regulatory tyrosine phosphorylation site, p50ck catalyzes phosphate transfer specifically to the COOH-terminal tyrosines of p59ftn, p56ck, and p59ftn (11). Moreover, in csk-deficient fetal mice (generated by targeted gene disruption in embryonic stem cells) the activities of p60ck and p59ftn 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 p50ck 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 p50ck can be detected at low levels in al-
most all adult mouse tissues; however, it is predominantly expressed in adult thymus and spleen. This restricted expression pattern suggests that p50 is 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 to and p59 to enhance the signaling process (18).

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

To evaluate further the in vivo role of p50, we have generated chimeric mice by injection of normal and RAG (recombination activating gene)-deficient blastocysts with embryonic stem (ES) cells bearing a homozygous disruption of csk. In this system, the developmental potential of the csk ES 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 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 ES cells proved perfectly capable of supporting myeloid cell development. These observations demonstrate that p50 acts to regulate differentiative events beginning at the time of lymphoid specification.

Materials and Methods

Generation of csk 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′-TATCGCTTCCTTGGACGATCTTCTGAGGG) and a second primer from within the targeted gene outside the disruption vector (5′-GCCCTCAGTGGCACCACAAGGCCCTAT). An additional 5′ primer (5′-TCCACCGGAGGCTTTTG-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 PCK 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 μg/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 blastochysms 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 mouse colony was established from breeders generously provided by Dr. Frederick W. Alt (Harvard Medical School, Boston, MA) (20). Blastocystics 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 antisera specific for p50 (12). Immunoreactive proteins were detected using a donkey anti-rabbit peroxidase-conjugated secondary antibody, and visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL) followed in some cases by densitometry (model UAS; Isis Lincoln, NE).

Semi-quantitative PCR. Genomic DNA was prepared by homogenization of tissue culture cells or whole organs in 100 mM Tris-Cl, pH 7.5, 10 mM MmCl2, 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 per unit of p60 protein present.

Immunoprecipitation and Kinase Assays. Immunoprecipitation of p60 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 MmCl2, 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 per unit of p60 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 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 ES 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′-TGGAGGGACAGGGTGCCAGGACCGGGCTAT). The incorporation of 32P was quantitated using the BAS2000 image analyzing system or autoradiography.

Immunocytochemistry. 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-Thyl.2FITC (PharMingen, San Diego, CA), and anti-B220PE (Caltag Labs, San Francisco, CA). Detection of biotinylated reagents was facil-
rated 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 chimeraic 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 × 10^5 and 1 × 10^5 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 mg/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 µmol 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\^~k, and for alterations in the specific activity of p60\^src. Fig. 2 A shows that the level of p50\^~k protein in ES cells directly reflects csk gene dosage; csk \(+/-\) cells express 50% less p50\^~k protein compared with wild-type cells, while csk\^null cells contain no immunoreac-
Figure 2. Absence of p50\(^{ak}\) and activation of p60\(^{c-src}\) in csk\(^{null}\) ES cells. (A) Immunoblot analysis reveals no p50\(^{ak}\) protein in whole cell lysates isolated from csk\(^{null}\) ES cells (lane 3) as compared with levels of p50\(^{ak}\) 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 \(\gamma^{32P}\)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". Moreover, the specific activity of p60" immu-
noprecipitated from csknull ES cells increased at least sixfold as compared with the measured activity in wild-type ES cells when judged either by autophosphorylation, or by phosphory-
lation of an exogenous substrate (Fig. 2 B). There was a similar increase in the specific activity of p59fr in csknull ES cells (data not shown). Interestingly, the amount of p60" protein in csknull ES cells was markedly less than in wild-type cells (Fig. 2 C), an effect previously observed in csknull embryos (15). Thus by direct evaluation these csknull ES cells do not express a functional csk gene product.

csknull ES Clones Do Not Support Development of Adult Lym-
phocytes. To determine if p50" is required for the normal
development of lymphoid cells, we produced chimeric mice
using ES cells lacking both alleles of p50". Three indepen-
dently-derived csknull 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 heterozy-
gote 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 leuk-
cytes expressed the Ly9.1 marker (data not shown). Thymo-
cytes and splenocytes from these mice were analyzed by three-
color flow cytometry to ensure that normal subpopulations

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**Figure 3.** Three-color flow cytometric analysis of lymphocytes and lymphocyte progenitors from adult csk+/− and csknull chimeric mice. (A) Three-color flow cytometry was performed on thymocytes and sple-
nocytes 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 per-
formed 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, spleno-
cytes, and bone marrow cells isolated from 4-wk-old csknull 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 ex-
pression 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 csknull 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 csknull ES cells, host precursors appeared to differentiate normally, indicating that the presence of the csknull stem cells had little effect on the development of endogenous wild-type progenitors (see below). Moreover, since the defect in the csknull 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 csknull ES Cells to Other Mouse Tissues. To assess the ability of csknull 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 csknull chimeric animals revealed that csknull 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 csknull-derived genomic DNA, such that heart (8% chimerism) and brain (5% chimerism) invariably contained the largest representation of csknull 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 csknull ES cells. However the overall low level of ES cell contribution found in the adult animals demonstrates that the developmental potential of csknull 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 csknull 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 csknull ES clones with those observed after injection of control cells, including wild-type ES cells, csk+/- ES cell clones, and fynnull ES cell clones (which support normal embryogenesis; 6). The csknull 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 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.

csknull ES Clones Can Develop into Immature Thymocytes in Fetal Chimeric Mice. Since csknull ES cells appeared capable of contributing to many cell populations, at least at low levels, we asked whether csknull 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 csknull chimaera examined at day 19 of gestation. In this animal, 5.8% of the thymocytes were derived from the csknull 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+ 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 CD440 cells, which may represent an early developmental potential of ES cells with this mutation. We have compared the litter sizes of chimeric mice made from the injection of csknull ES clones with those observed after injection of control cells, including wild-type ES cells, csk+/- ES cell clones, and fynnull ES cell clones (which support normal embryogenesis; 6). The csknull 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 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.

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Figure 5. csk<sup>+</sup> 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<sup>+</sup> chimeric mice. Cells were stained with combinations of anti-CD4<sub>PE</sub> and anti-CD8<sub>FITC</sub>, anti-TCR-β<sub>PE</sub> and anti-CD3<sub>FITC</sub>, 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-β staining of thymocytes gated on Lyg.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<sup>b</sup> population. Very few mature cells, expressing CD3<sub>e</sub>, were represented among the Ly9.1<sup>b</sup> cells (Fig. 5 D). This inability of csk<sup>-</sup> stem cells to yield phenotypically mature intrathymic progeny was confirmed in fetal day 17 chimeric mice (data not shown).

Reconstitution of RAG-2<sup>-</sup> Blastocysts with csk<sup>-</sup> ES Clones. Although the differentiative capability of csk<sup>-</sup> T cell progenitors appeared to be severely compromised, it was possible that this phenotype resulted from inefficient competition with wild-type T lineage cells in the chimeric thymus. To examine more directly the innate ability of csk<sup>-</sup> intrathymic precursors to give rise to T cell progeny, we generated additional chimeras using RAG-2<sup>-</sup> blastocysts as hosts. In these animals, thymopoiesis arrests at the CD4<sup>+</sup>-8<sup>−</sup>, IL-2Rα<sup>+</sup> stage (19, 24). Hence this environment should permit development of csk<sup>-</sup> cells unhindered by host cell competition. Nevertheless, as in the chimeras generated using wild-type blastocysts, csk<sup>-</sup> ES cells proved incapable of contributing to the development of adult lymphoid populations.
Control Thymus  | CSK−/− RAG 2 null chimeric | RAG 2 null control
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CD3 | CD4 | CD8

Control Thymus: 2.3% CD3+ 90.4% CD4- 2.8% CD8-
CSK−/− RAG 2 null chimeric: 3.9% CD3+ 5.1% CD4- 3.5% CD8-
RAG 2 null control: 0.05% CD3+ 0.2% CD4- 0.1% CD8-

Figure 6. Reconstitution of RAG-2null 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-2null blastocysts reconstituted with csk−/− ES cells, and RAG-2null controls. Histograms of CD4 vs. CD8 and CD3 vs. anti-TCR-δ are shown.

(data not shown). Analysis of csknull/RAG-2null chimeric mice at fetal day 19 once again defined the limits of intrathymic differentiative capability for csknull cells. Immature CD8+ and CD4+8+ 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 CD3lo population, that which ordinarily represents about half of all CD4+8+ cells (Fig. 6). Even γ/δ T cells, which share a very early progenitor with α/β T cells (25), fail to develop from csknull precursors (Fig. 6). Again, a relatively large number (3.9% of the total) of CD4lo cells, not immediately related to any normally abundant thymocyte population, emerges in the csknull 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 csknull Precursors. The inability of csknull progenitors to give rise to normal T or B lymphocytes (Fig. 3 B) suggested that csk function might

Figure 7. Development of granulocyte-macrophage lineage cells in CFU-GM assays. CFU-GM assays were performed on fetal liver cells obtained from fetal day 17 csknull chimeric mice. (A) Analysis of a colony subsequently shown by PCR to contain only csknull 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 csknull 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 csknull 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 csknull ES clones to develop within the myeloid linkage, single cell suspensions derived from livers of csknull 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 csknull origin. These csknull 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 csknull 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, p60csrc itself must be expressed to permit normal osteoclast development (27), whereas a deficiency of p56lck 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 p56lck 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 p56lck activity suppresses rearrangement of Vβ gene segments, promotes rearrangement of Vα gene segments, and simultaneously drives the proliferative expansion and maturation of CD4+8+ cells (30). Activation of p56lck can be stimulated through dephosphorylation of Tyr505, the site of tyrosine phosphorylation (31). Since Tyr505 is also the principle site of tyrosine phosphorylation of p56lck in vivo (31, 32), there was reason to believe that p50src might serve as a component of the regulatory machinery that ordinarily permits activation of p56lck in response to TCR-β chain expression.

The p50src 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 csk (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, p50src, 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 p56lck. In its simplest form, the hypothesis that p50src acts to regulate p56lck 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 p50src plays a much more fundamental role in regulating lymphocyte development.

Several important features of p50src function were illuminated by examining chimeric mice generated using csknull ES cells. First, ES cells lacking p50src protein are indistinguishable from wild-type cells with respect to in vitro growth properties, even though the activity of p60csrc in such cells is significantly elevated (Fig. 2). Thus, p50src 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 csknull 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 csknull ES cells rather than the host blastomeres.

One intriguing feature of the csknull 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 p50src effects can be seen most clearly in hematopoietic populations. Precursors derived from csknull 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 csknull 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 p50src controls lymphopoiesis), permitting appropriate differentiation even in a csknull background. Importantly, both T and B cell generation are effectively stymied in chimeras made using csknull ES cells, implying either that p50src 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 p50src activity.

Regardless of the site of action of p50src in lymphopoiesis, the characteristics of the defect observed in csknull thymocytes permit important conclusions regarding its function. We initiated these studies with the expectation that p50src might participate in regulating p56lck or p59fr. How-
ever inappropriate activation of p56\(^{ck}\) promotes, rather than suppresses, the development of CD4\(^+\)8\(^+\) thymocytes, and activated p59\(^{F}nk\), 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\(^{ck}\) acts on targets other than the src-family kinases known to regulate T cell development. The recent observation that p50\(^{ck}\) can phosphorylate and activate the CD45 phosphotyrosine phosphatase supports this view, in the sense that there exist demonstrable substrates for p50\(^{ck}\) other than src-family kinases.

What can be said of the intrathymic process regulated by p50\(^{ck}\)? 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\(^+\)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\(^+\) cells, but that differentiation thereafter proceeds very poorly, since the representation of CD4\(^-\)8\(^-\), CD4\(^-\)8\(^b\), and CD4\(^+\)8\(^+\) cells is far less than what would be expected given the abundance of the CD4\(^+\)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\(^{ck}\) controls p56\(^{ck}\) activity and hence the maturation of CD4\(^+\)8\(^+\) cells from CD4\(^+\)8\(^-\) thymoblasts. Instead, since p50\(^{ck}\) clearly regulates an even earlier developmental transition, the involvement of p50\(^{ck}\) in modulating p56\(^{ck}\) activity could not be addressed. Detailed analysis of the stage-specific functions of p50\(^{ck}\) 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\(^{ck}\) is selectively required for the satisfactory maturation of early lymphoid progenitors. Elucidation of the mechanism whereby p50\(^{ck}\) 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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