Cytokine Signaling and Hematopoietic Homeostasis Are Disrupted in Lnk-deficient Mice

Laura Velazquez,1 Alec M. Cheng,3 Heather E. Fleming,4,5 Caren Furlonger,1 Shirly Vesely,1 Alan Bernstein,1,2,6 Christopher J. Paige,4,5 and Tony Pawson1,2

1Samuel Lunenfeld Research Institute, Mount Sinai Hospital and 2Department of Medical and Molecular Genetics, University of Toronto, Toronto, Ontario M5G 1X5, Canada
3Division of Rheumatology, Washington University School of Medicine, St. Louis, MO 63110
4Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, and 5Departments of Immunology and Medical Biophysics, University of Toronto, Toronto, Ontario M5G 2M9, Canada
6The Canadian Institutes of Health Research, Ottawa, Ontario K1A 0W9, Canada

Abstract

The adaptor protein Lnk, and the closely related proteins APS and SH2B, form a subfamily of SH2 domain-containing proteins implicated in growth factor, cytokine, and immunoreceptor signaling. To elucidate the physiological function of Lnk, we derived Lnk-deficient mice. Lnk−/− mice are viable, but display marked changes in the hematopoietic compartment, including splenomegaly and abnormal lymphoid and myeloid homeostasis. The in vitro proliferative capacity and absolute numbers of hematopoietic progenitors from Lnk−/− mice are greatly increased, in part due to hypersensitivity to several cytokines. Moreover, an increased synergy between stem cell factor and either interleukin (IL)-3 or IL-7 was observed in Lnk−/− cells. Furthermore, Lnk inactivation causes abnormal modulation of IL-3 and stem cell factor–mediated signaling pathways. Consistent with these results, we also show that Lnk is highly expressed in multipotent cells and committed precursors in the erythroid, megakaryocyte, and myeloid lineages. These data implicate Lnk as playing an important role in hematopoiesis and in the regulation of growth factor and cytokine receptor–mediated signaling.

Key words: knockout • hematopoiesis • cell proliferation • growth factors • signal transduction

Introduction

The growth, differentiation, and function of immune and hematopoietic cells are controlled by the coordinated actions of multiple cytokines (1). Members of the cytokine receptor superfamily function through their association with one or more members of the Janus (JAK)* family of cytoplasmic tyrosine kinases (2, 3). Cytokines induce oligomerization of their receptors, followed by transphosphorylation and activation of the JAK kinases. The activated JAKs phosphorylate tyrosine residues in the receptor and subsequently downstream substrates, such as the signal transducers and activators of transcription (STAT) proteins (4). Once recruited to the receptor complex, STAT proteins are themselves phosphorylated on tyrosine, and dimerize and translocate into the nucleus, where they activate the transcription of genes mediating cytokine-induced responses. The JAK/STAT pathway is tightly regulated, and deregulated JAK or STAT activity has been implicated in several hematopoietic disorders, autoimmunity, inflammatory diseases, and in cell transformation (5–8).

Cytokines also activate other signaling cascades, such as the Ras/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways (9–11). These have been implicated in the proliferation, survival, and differentiation of several cell types in the hematopoietic system (12–14). Indeed, deregulation of these pathways has been implicated in the pathogenesis of hematopoietic diseases (15–18).

*Abbreviations used in this paper: BCR, B cell receptor; BMMC, bone marrow–derived mast cell; EMH, extramedullary hematopoiesis; EPO, erythropoietin; ES, embryonic stem; FACS, flow cytometric analysis; IL-3Rβ, IL-3 receptor β chain; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; pBCR, pre-B cell receptor; PH, pleckstrin domain; PI3K, phosphoinositide 3-kinase; SCF, stem cell factor; SH2, src homology 2; STAT, signal transducer and activator of transcription.
Lnk was originally identified as a 38-kD adaptor protein expressed mainly in hematopoietic tissues (19, 20). However, it was later found that the Lnk protein is much larger than initially reported (21, 22, and this work). The full-length mouse Lnk protein contains an NH2-terminal proline rich region, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and potential tyrosine phosphorylation sites. Lnk, and the closely related proteins APS and SH2-B, form a subfamily of SH2-containing proteins with potential adaptor functions (23, 24). Members of the Lnk family are expressed in a wide variety of tissues, and therefore, may be involved in diverse signaling pathways. Indeed, APS and SH2-B have been shown to be phosphorylated after stimulation of growth factor (23, 25–30), cytokine (23–33), and immunoreceptors (21, 23, 24). SH2-B (an isoform of SH2-B) and APS are substrates of the Trk family of neurotrophin receptors, and positive mediators of nerve growth factor signaling in PC12 cells (27).

SH2-B is also an activator of JAK2 in response to growth hormone (34), whereas APS appears to be a negative regulator of JAK2 after erythropoietin (EPO) stimulation (32) and inhibits platelet-derived growth factor–induced mitogenesis (35). These observations suggest that these adaptor proteins are regulators of growth factor and cytokine receptor-mediated pathways.

Despite substantial data implicating this new family of adaptor proteins in signaling, their physiological roles in hematopoietic cell signaling have not been defined. However, Takaki et al. (22) have demonstrated that Lnk-deficient mice display defects in B lymphopoiesis. We have also generated Lnk-deficient mice and have also observed an effect on B cell production and function. In addition, we demonstrate here that Lnk−/− mice display splenomegaly, hyperplasia of the megakaryocytic lineages, and increased numbers of both erythroid cells in the spleen and hematopoietic progenitors. Consistent with this, we show that Lnk is highly expressed in multipotent cells and precursors of hematopoietic lineages. Moreover, Lnk−/− cells show an increased sensitivity to several cytokines and altered activation of the Ras/MAPK pathway in response to IL-3 and stem cell factor (SCF). These results demonstrate that Lnk regulates proliferation of several hematopoietic cell lineages and suggest that it is an important component of growth factor and cytokine signal transduction in hematopoietic cells.

**Materials and Methods**

**Generation of Lnk-deficient Mice.** A 129/Sv mouse genomic library (Stratagene) was screened with a rat Lnk cDNA probe. Exon/intron boundaries were determined by restriction enzyme mapping, DNA sequencing, and PCR. To generate the targeting construct, a 5-kb SacI fragment containing 5′ Lnk genomic DNA and a 2-kb SacI fragment containing 3′ sequence were cloned into pPNT. The targeting construct was linearized with NotI and electroporated into R1 embryonic stem (ES) cells, and transfectants were selected as described previously (36, 37). Aggregation chimeras were generated with targeted ES cells, and chimeric males were mated with 129/Sv females and germine transmission of the mutated allele was verified by PCR analysis of tail DNA from F1 mice. Heterozygous offspring were intercrossed to generate homozygous mutants. No differences in phenotype were observed between Lnk mutant mice from different genetic backgrounds (129/Sv or C57BL/6).

Poly(A)+ RNA from E13.5 embryos from each genotype was purified once on an oligo-(dT) column. 2 μg of poly(A)+ RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech). Hybridization was performed in Church buffer (500 mM phosphate buffer, pH 6.8, and 7% SDS) at 65°C overnight. A 500-bp NH2-terminal Lnk cDNA fragment was used as a probe. The membrane was washed in 0.1× SSC, 0.1% SDS at 65°C. The Northern blot was stripped by boiling for 5 min in 0.1% SDS, 0.1× SSC, and 0.05% sodium pyrophosphate. RNA loading was evaluated by hybridization with a 1.4-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

**Immunohistochemistry and Peripheral Blood Analysis.** Mouse tissues were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. For immunohistochemical analysis, spleens were embedded in OCT compound (Tissue-Tek Sakura), snap frozen in dry ice, and stored at −70°C. Cryostat sections (8–10 μm thick) were dried overnight and fixed with acetone. Slides were rehydrated and preincubated for 1 h in staining buffer (1× PBS and 0.1% NP-40) containing 5% heat-inactivated rat serum. Sections were incubated with FITC- or phycoerythrin (PE)-conjugated primary antibodies (BD PharMingen) in a humidified chamber for 1 h at room temperature. After three washes in staining buffer and one wash in 1× PBS, the slides were mounted in p-phenylenediamine (Sigma–Aldrich) in PBS and glycerol (50% vol/vol) to retard photobleaching. The antibodies used for immunohistochemistry were PE-anti-B220, PE-anti-Ter119, and FITC-anti-CD41. Visual data were acquired with a fluorescent microscope (Leica).

Peripheral blood analysis was performed on a blood analyzer (Coulter electronics). Peripheral blood smears were stained with Wright-Giemsa (Fisher) and scored microscopically based on morphology.

**Flow Cytometric Analysis.** Bone marrow and spleen cells obtained from wild-type or mutant mice were made into single cell suspensions in staining buffer (cold PBS supplemented with 2% FBS). The cells were stained on ice for 30 min with FITC, PE, APC, or biotin-conjugated mAbs. The stained cells were washed twice in staining buffer and analyzed using a FACSscan™ flow cytometer and CellQuest™ software (Becton Dickinson). The following mAbs were used: PE, APC, or biotin–anti-B220, PE–anti-Ter119, PE–anti-Mac-1, PE–anti-CD43, PE–anti-CD22, PE–anti-CD23, FITC–anti-CD41, FITC–anti-Gr-1, FITC–anti-BP-1, and biotin–anti-HSA; all were purchased from BD PharMingen. FITC–anti-IgM is the clone 33.60 (38). Biotin–stained cells were labeled with Quantum red (Sigma–Aldrich).

**B Cell Proliferation Assays.** To obtain enriched B cell populations, splenocytes from wild-type and mutant mice were treated with ACK (0.155 M ammonium chloride, 0.1 mM disodium EDTA, and 0.01 M potassium bicarbonate) for red cell lysis followed by T cell depletion. Cells were incubated with anti-Thy1.2 antibody (clone H103.4.9), anti-CD4 (clone RL172.4H), and anti-CD8 (clone 3.168) supernatants and low tox guinea pig complement (Cedarlane Laboratories). The resulting enriched splenic B cells were cultured at 105 cells/well in flat-bottom 96-well plates in OPTI-MEM (GIBCO BRL) medium + 5% FBS.
and with or without 100 ng/ml IL-7, 50 ng/ml SCF (Peprotech), IL-7 and SCF, 10 μg/ml LPS, and 10 μg/ml anti-IgM for 2–6 d. The cultures were pulsed with 1 μCi/well [3H]thymidine for 6 h and harvested. Incorporated radioactivity was determined by a Betaplate Counter (Packard Instrument Co.). IL-7 was obtained from the supernatant of the stably transfected J558 line (Dr. A Cumano, Institute Pasteur, Paris, France). For the proliferation assay of CD43+ pro-B cells, bone marrow cells were enriched and prepared as described previously (39).

In Vitro Bone Marrow and Spleen Cultures. For liquid cultures, single cell suspensions of bone marrow and spleen cells were prepared in IMDM ( Gibco BRL) supplemented with 15% heat-inactivated FBS, 50 μM B-mercaptoethanol, antibiotics, 200 μg/ml transferrin (Sigma-Aldrich), 10 μg/ml insulin (Boehringer), and 1% BSA. Cells were plated in 96-well tissue culture plates at 104 (for bone marrow) or 5 × 104 cells/well (for spleen) in various concentrations of recombinant murine IL-3 (Peprotech), SCF (Peprotech), or IL-3 and SCF. After 4–6 d in culture, cells were cultured with MTT [3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide] as suggested by the manufacturer (Boehringer).

In Vitro Hematopoietic Progenitor Colony Assays. Single cell suspensions were prepared from bone marrow aspirates or spleen under sterile conditions. Triplicate samples of 104 or 105 cells (for CFU-GEMM) were plated in methylcellulose (MethoCult M3434) containing cytokines, including erythropoietin (Stem Cell Technologies Inc.), and incubated at 37°C, 5% CO2, 100% humidity. Progenitor colony-forming units were counted at 2 d (CFU-E), 8 d (BFU-E), and 10 d (CFU-GM, CFU-GEMM) after benzidine staining. Megakaryocyte progenitors (2 × 103 cells) were assayed in collagen-based media (MegaCult-C; Stem Cell Technologies Inc.). Colonies were stained for acetylcholinesterase activity and counted after 6 d incubation. Bone marrow pro-B cell progenitors (5 × 104 cells) were detected by culture in methylcellulose media (MethoCult M3231; Stem Cell Technologies Inc.) supplemented with IL-7 (10 ng/ml; BD PharMingen) or IL-7 and SCF (50 ng/ml; Peprotech). Colonies were scored microscopically after 7 d.

Immunoprecipitation and Western Blot Analysis. Polyclonal rabbit antiserum was raised against an NH2-terminal synthetic peptide (amino acid 72–91) of mouse Lnk. Spleens and testis from wild-type, heterozygous, and homozygous mice were homogenized and lysed in ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10% glycerol, 1 and prepared as described previously (39).

In Vitro Bone Marrow and Spleen Cultures. For liquid cultures, single cell suspensions of bone marrow and spleen cells were prepared in IMDM (Gibco BRL) supplemented with 15% heat-inactivated FBS, 50 μM B-mercaptoethanol, antibiotics, 200 μg/ml transferrin (Sigma-Aldrich), 10 μg/ml insulin (Boehringer), and 1% BSA. Cells were plated in 96-well tissue culture plates at 104 (for bone marrow) or 5 × 104 cells/well (for spleen) in various concentrations of recombinant murine IL-3 (Peprotech), SCF (Peprotech), or IL-3 and SCF. After 4–6 d in culture, cells were cultured with MTT [3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide] as suggested by the manufacturer (Boehringer).

Results

Disruption of the Lnk Gene and Generation of Lnk−/− Mice. To address the biological function of Lnk in vivo, we generated mice carrying a germline mutation in Lnk by homologous recombination in RI mouse ES cells. A targeting vector was designed to replace a 1.6-kb DNA fragment containing exons II–VII of Lnk with a PGK-neomycin cassette (Fig. 1 A). The deleted exons encoded the originally reported coding sequence of Lnk (19). Successful homologous recombination and germ line transmission was confirmed by Southern blot and PCR analysis of ES origins (offspring of heterozygous parents), respectively (Fig. 1, B and C). Northern and Western blot analyses of different tissue extracts derived from mice of each genotype were performed with a 500-bp NH2-terminal Lnk probe (exon 1) or antibodies raised against the NH2-terminal region of the Lnk protein, respectively (Fig. 1, D and E). This analysis confirmed the absence of Lnk-specific protein products in the Lnk−/− deficient mice.

Lnk−/− Mice Exhibit Extramedullary Hematopoiesis. Homozygous Lnk−/− mice were present at the expected Mendelian ratio of 1:2:1 (n = 330) from intercrosses of heterozygous parents. Both Lnk−/− female and male animals were viable and fertile, with normal longevity.
We performed a histological analysis of neonatal and adult mice to identify tissue abnormalities in Lnk-deficient animals. This identified a severe splenic disorder in Lnk−/− mice characterized by splenomegaly (weight, 0.062 ± 0.015 g for +/+ mice compared with 0.195 ± 0.07 g for −/− mice; P < 0.01; Fig. 2 A), fibrosis, and extensive extramedullary hematopoiesis (EMH), in which increased numbers of megakaryocytes (CD41+ cells) and erythrocytes (Ter119+ cells) were apparent in the splenic red pulp (Fig. 2, C, F, and G). This increased EMH in the spleen of mutant mice was apparent from birth (unpublished data). In general, the architecture of the spleen was considerably changed in the Lnk-deficient mice. The white pulp, with its characteristic discrete lymphoid follicles (Fig. 2 B), presented dramatic changes in shape (Fig. 2 C). Lymph nodes were often enlarged in Lnk−/− mice and showed increased numbers of B cells (unpublished data). No morphological differences between wild-type and Lnk-deficient animals were observed in any other tissue, including thymus, liver, kidney, and lungs. For all parameters analyzed, the Lnk heterozygous mice showed an intermediate phenotype between the wild-type and homozygous mutant mice (Fig. 2 A and unpublished data). Together, these results revealed hyperplasia of the megakaryocytic, erythroid, and B cell lineages in the spleen of Lnk−/− mice with the latter two contributing to increased cellularity in this organ and splenomegaly.
WBC, white blood cell count; RBC, red blood cell count.

Differential counts (10^6/ml)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+</th>
<th>−/−</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^9/ml)</td>
<td>7.6 ± 1.5</td>
<td>26.6 ± 6.04</td>
<td>**</td>
</tr>
<tr>
<td>RBC (10^6/ml)</td>
<td>8.4 ± 0.9</td>
<td>7.8 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48 ± 3.3</td>
<td>49 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Platelets (10^9/ml)</td>
<td>400 ± 107</td>
<td>1,903 ± 219</td>
<td>**</td>
</tr>
</tbody>
</table>

Differential counts (10^9/ml)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+a</th>
<th>−/−a</th>
<th>P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>6.44 ± 1.39</td>
<td>22.12 ± 5.55</td>
<td>**</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.130 ± 0.05</td>
<td>0.420 ± 0.102</td>
<td>**</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.04 ± 0.51</td>
<td>3.91 ± 1.15</td>
<td>**</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.106 ± 0.07</td>
<td>0.394 ± 0.22</td>
<td>*</td>
</tr>
</tbody>
</table>

Table I. Hematological Parameters of Wild-type and Lnk-deficient Mice

Table II. Cell Population in Wild-type and Lnk-deficient mice (in millions)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/+a</th>
<th>−/−a</th>
</tr>
</thead>
</table>

Lnk−/− Mice Exhibit Defects in Hematopoietic Homeostasis. Based on these observations, we examined specific hematological parameters in the mutant mice. No significant differences in peripheral red blood cell counts or in hematocrits were observed between wild-type and Lnk−/− mice. However, the white blood cell counts and circulating platelet levels in Lnk mutants were markedly increased as compared with wild-type mice. Differential counts of peripheral blood smears revealed a substantial elevation in the numbers of circulating lymphocytes, monocytes, and neutrophils (Table I).

Flow cytometric analysis (FACS) of bone marrow and spleen cells was then performed to further characterize the hematopoietic perturbations in the Lnk−/− mice. The percentage of B220+ lymphocytes was increased in the bone marrow and in the spleen (8–13% above +/+ littermates) of Lnk-deficient mice (Table II). Similarly, the percentage of cells positive for CD41, expressed on megakaryocytes, was elevated in the bone marrow (twofold) and more dramatically in the spleen (threefold) of Lnk−/− mice (Table II). In addition, the percentage of Ter-119–positive erythroid cells in Lnk-deficient mice was decreased 36% in the bone marrow but increased twofold in the spleen of Lnk mutant mice (Table II), which is consistent with the elevated splenic erythropoiesis observed previously (Fig. 2A). Finally, the percentage of Gr-1/Mac-1+ cells, representing monocyte/granulocyte progenitors, was decreased 15% in the bone marrow, but remained similar in the spleen of Lnk−/− mice (Table II). When combined with the increase in cellularity in both the bone marrow and spleen compartments of Lnk−/− mice, these FACS data reveal a significant elevation of both B lymphoid and megakaryocyte populations, as well as enhanced erythroid numbers in the spleen (Table II). The FACS data are consistent with the changes observed in the peripheral blood counts and with the histological findings in bone marrow and spleen of Lnk-deficient mice.

Lnk−/− Mice Have Altered Hematopoietic Progenitor Numbers. To determine if the perturbations in the hematopoietic organs and peripheral blood arise at the progenitor level, bone marrow and spleen cells from Lnk+/+ and Lnk−/− mice were grown in vitro in semi-solid media with various growth factors to quantitate multilineage (CFU-GEMM), pre-B, megakaryocytic (CFU-Meg), and erythroid (BFU-E and CFU-E) progenitor cells (Fig. 3A). The absolute numbers of clonogenic progenitors observed with Lnk+/+ cells were intermediate between wild-type and mutant levels (unpublished data). The number of multilineage colony-forming cells (CFU-GEMM) was significantly increased in Lnk−/− bone marrow (2.5-fold) and spleen (8-

Table II. Cell Population in Wild-type and Lnk-deficient mice (in millions)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total cells</th>
<th>B cells B220+</th>
<th>Erythroid Ter119+</th>
<th>Megakaryocyte CD41+</th>
<th>Myeloid Gr-1/Mac-1+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
<td>No. %</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>13 ± 3.6</td>
<td>4.3 ± 0.59</td>
<td>34.1 ± 4.6</td>
<td>0.90 ± 0.20</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>Lnk-deficient</td>
<td>14 ± 1.2</td>
<td>6.6 ± 0.71</td>
<td>47.5 ± 5.1</td>
<td>0.63 ± 0.20</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>63 ± 15.4</td>
<td>32.1 ± 5.4</td>
<td>51 ± 8.6</td>
<td>2.2 ± 0.98</td>
<td>3.5 ± 1.6</td>
</tr>
<tr>
<td>Lnk-deficient</td>
<td>82 ± 25</td>
<td>49 ± 3.1</td>
<td>59 ± 3.8</td>
<td>4.6 ± 0.98</td>
<td>6.0 ± 1.2</td>
</tr>
</tbody>
</table>

The phenotype of each cell population was determined by flow cytometry. The numbers and percentage of each group of cells were determined for each mouse for at least three to five mice per genotype, and the mean and SD were calculated.

a. Values represent the mean ± SD for 10 animals per determination per genotype.
b. Statistical significance was determined using the Student’s t test where *, P ≤ 0.05; **, P ≤ 0.005; NS, not significant.

1603 Velazquez et al.
fold) compared with +/+ littermates. Similarly, the number of pre-B lymphoid colony-forming cells in Lnk−/− bone marrow was increased two- and sevenfold relative to wild-type mice in the presence of IL-7 or IL-7 and SCF, respectively (Fig. 3 A). Although only slight differences were observed in the number of precursors of small erythroid 2-d colonies (CFU-E) and CFU-megakaryocyte (CFU-Meg) in Lnk−/− bone marrow, increased numbers of these colonies were seen in cells derived from the spleen. A similar result was observed with precursors of large erythroid-only 6–8-d colonies (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor numbers (unpublished data).

Moreover, there was a marked difference in the proliferative capacity of Lnk−/− progenitors. The pre-B lymphoid and CFU-Meg colonies from bone marrow and spleen of Lnk-deficient mice were typically three to five times larger than the colonies derived from wild-type mice (Fig. 3 B). We observed a similar effect on the colony size of CFU-GEMM from Lnk−/− mice. These results suggest that the variations in differentiation potential observed are intrinsic to the Lnk−/− progenitor cells, and that these cells have an enhanced responsiveness to cytokines.

**Lnk−/− Mice Are Affected in B Cell Maturation and Proliferation.** Because there was an increase in the number of B220+ cells in the Lnk mutant mice, we examined which B cell populations were affected by the lack of the Lnk protein. FACS analysis of bone marrow–derived B cells revealed that wild-type and Lnk mutant mice display similar percentages of pro-B (B220+CD43+) cells (Fig. 4 A). Further analysis of the pro-B compartment by four-color FACS staining indicated that Lnk−/− mice have an increase in the percentage of BP.1+HSA+ cells relative to wild-type mice (Fig. 4 A). This population corresponds to the IL-7–responsive, Fraction C stage of B cell development (43). The increase in Fraction C cells is accompanied by a slight decrease in the percentage of HSA+BP.1−, Fraction B cells (Fig. 4 A). A more profound increase is observed in the pre-B cell population in Lnk-deficient animals, which can be visualized by the B220+IgM− and B220+CD43+ populations shown in Fig. 4 A. Later stages of development were also altered in the absence of Lnk expression. An accumulation of the immature population of B220+CD43−IgM+ cells and a decreased percentage of more mature, recirculating B220+IgM+ cells were identified in Lnk-deficient mice (Fig. 4 A). In addition, a greater proportion of the IgM+ cells in the bone marrow and spleen of Lnk−/− mice expressed reduced levels of CD22 and CD23, which is consistent with the observed accumulation of cells at the immature B cell stage of development (Fig. 4, A and B). The differences in the proportion of B cell precursors in wild-type and Lnk-deficient animals are more pronounced when the increase in Lnk−/− bone marrow cellularity is taken into account. This analysis also reveals that the absolute number of recirculating cells is similar to that found in control mice (1.5-fold increase, compared with 3–4-fold increase in pro-, pre-, and immature B cells; unpublished data). Elevated numbers of immature IgM+ B cells may arise after the maturation of the expanded pre-B population present in Lnk−/− mice, or may indicate incomplete maturation past the B220+CD43−IgM−, Fraction E cell stage in the absence of Lnk expression. Support for the hypothesis of incomplete maturation of the B cell lineage was found in the spleen, where the presence of a high frequency of IL-7–responsive cells was observed (1:100 in Lnk−/− compared with 1:11,000 in wild-type mice; unpublished data) in the absence of detectable numbers of IgM− B cell precursors (Fig. 4 B). Together, these data indicate that an increase in B lineage cells is evident as early as the IL-7–responsive, Fraction C stage of development, and that an accumulation of cells at the...
B220low, immature B cell stage occurs in the bone marrow and spleen in Lnk-deficient mice.

To further assess the maturation state of B cell precursors in the Lnk-deficient mice, we analyzed the ability of bone marrow and splenic B cells to proliferate in response to various stimuli. CD43+/H11001 pro-B cells were sorted from the bone marrow and grown in a titration of IL-7. These culture conditions permit the expansion of IL-7-responsive pro-B cells, as well as their maturation to the pre-B cell receptor (pBCR)–expressing pre-B stage. Recent reports have demonstrated that a signal downstream of the pBCR is required for proliferation in picogram concentrations of IL-7 (39). As shown in Fig. 5 A, cells from both the Lnk−/− and control mice are able to proliferate above background in low concentrations of IL-7. A slight elevation in background [3H]thymidine incorporation by Lnk-deficient cells is observed in the absence of IL-7. However, these cells exhibit a more significant twofold elevation in proliferation when stimulated with picogram per milliliter concentrations of IL-7. FACS staining revealed that cells from both geno-
types matured to express CD2 in high and low concentrations of IL-7 (unpublished data). The absence of a significant increase in the proliferation of mutant precursors in response to nanogram concentrations of IL-7 suggests that the phenotype observed in reduced levels of IL-7 is not due to the presence of a higher percentage of BP.1+ cells in the sorted population. Together, these results indicate that Lnk expression may not be required to transmit pBCR signals at this stage in development, and suggest that the pre-B cells have not fully matured and lost the ability to respond to IL-7 (44). Similarly, splenic B cells from Lnk-deficient mice showed a significant increase in proliferation after IL-7 stimulation (Fig. 5 B). This is consistent with the elevated frequency of IL-7-responsive precursors detected in the mutant spleen, and is indicative of a very immature phenotype extending to the periphery. In addition, the proliferation of Lnk-deficient splenic B cells is further enhanced by concurrent stimulation with IL-7 and SCF (Fig. 5 B).

Splenic B cells were also assayed for proliferation after BCR stimulation. Although Lnk-deficient cells maintain the ability to proliferate in response to the mitogen LPS, the response of splenic Lnk−/− B cells to anti-IgM was partially reduced at any of the time points examined (Fig. 5 C and unpublished data). Cells at the immature B cell stage are unable to mount a proliferative response after BCR stimulation (45). This is consistent with the elevated numbers of immature B cells observed in the spleen (Fig. 4 B). Alternatively, Lnk may be required by the BCR to transmit a signal leading to proliferation. Further experiments are underway to examine these issues. Together, these results indicate that the B lineage cells derived from Lnk−/− mice maintain growth characteristics that are typical of immature populations. The B cell phenotype that we observed is similar to the one identified by Takaki et al. (22). However, in contrast with this group, we do not detect a striking difference between wild-type and Lnk-deficient mice in the mature B220+IgM+ population recirculating in the bone marrow from the periphery. Therefore, in our hands, the absence of the Lnk protein does not prevent the recirculation of mature B cells into the bone marrow compartment, but does lead to an accumulation of phenotypically immature B cells.

**Figure 5.** Functional analysis of Lnk−/− B cells. (A) Bone marrow sorted pro-B cells from wild-type and Lnk-deficient mice were cultured with the indicated concentrations of IL-7 and proliferation was measured at day 6 by [3H]thymidine incorporation. The values are the mean counts per minute (±SD) of triplicate determinations. (B and C) Splenic B cells were cultured with the indicated stimuli and proliferation was measured at day 6 (B) or day 4 (C) by [3H]thymidine incorporation. The values are the mean counts per minute (±SD) of triplicate determinations. All graphs are representative of independent experiments repeated three to five times. For each experiment, two mice per genotype were used.

**Lnk−/− Cells Exhibit Increased Proliferative Responses to Different Cytokines.** The results of the clonogenic and B cell proliferation assays suggest that Lnk may play an important role in regulating myeloid and B cell progenitor proliferation. SCF, in combination with other growth factors, such as IL-3, stimulates the proliferation of most hematopoietic progenitors and mature cells (46). Therefore, we examined the growth response of other hematopoietic cells derived from wild-type and Lnk−/− mice to various mitogenic stimuli. We first analyzed the response of primary bone marrow and splenic cells from wild-type and Lnk−/− mice to various concentrations of SCF, IL-3, or a combination of both factors. No difference in proliferation was detected between wild-type and Lnk−/− bone marrow cells with SCF or IL-3 alone (Fig. 6 A, left and middle). The addition of SCF to bone marrow cultures stimulated with IL-3 induced an increased expansion of Lnk−/− cells that was not seen in wild-type cells (Fig. 6 A, right). A similar result was observed when scoring the number of progenitor colonies formed in the presence of SCF and different concentrations of IL-3 from Lnk−/− mice (unpublished data). However, primary Lnk−/− splenocytes exhibited an enhanced sensitivity to either SCF or IL-3 alone relative to wild-type cells (Fig. 6 B). This hypersensitive proliferation of Lnk−/− cells was more pronounced in cultures stimulated with both SCF and IL-3 (Fig. 6 B). To pursue the effects of Lnk disruption in mitogenic signals, we examined the growth properties of wild-type and Lnk−/− BMMCs in response to SCF and IL-3. These cells provide a model system to study the effects of these cytokines on cell proliferation and survival in a homogeneous population. The mitogenic effects of SCF and IL-3 were characterized by determining dose-dependent DNA synthesis on SCF or IL-3 stimulation. Inactivation of Lnk resulted in increased incorporation rates of [3H]thymidine upon treatment of BMMCs with SCF (Fig. 6 C). However, no consistent difference was found between wild-type and Lnk-deficient BMMCs stimulated with IL-3 (Fig. 6 C). These observations suggest that Lnk negatively regulates the proliferative response to growth factors in hematopoietic cells of different origin and demonstrate that SCF alone or in synergy with IL-3 can induce a hypersensitive proliferative response in vitro in Lnk−/−-derived cells.
**Lnk**<sup>−/−</sup> Cells Show Defects in SCF and IL-3 Signaling. To assess the role of Lnk in the SCF and IL-3 pathways, we used BMMC from wild-type and Lnk-deficient mice. These cells express both Kit, the receptor for SCF, and IL-3R chains, and their responses to IL-3 and SCF have been well characterized (47, 48). Cells from both Lnk-deficient and wild-type mice were starved for 12 h and then treated for 10 min with IL-3. Both types of cells showed a similar activation of the IL-3Rβ chain after IL-3 stimulation as judged by antiphosphotyrosine blotting (Fig. 7 A). We next examined the kinetics and extent of MAPK and Akt activation on IL-3 stimulation of BMMCs. Cells were starved and stimulated at different times with IL-3. No differences in the activation of Akt were detected between wild-type and Lnk<sup>−/−</sup> BMMCs (Fig. 7 A). However, the analysis of MAPK activation showed that the kinetics of ERK1/2 were altered, such that the peak activation of ERK1/2 was slightly delayed in Lnk<sup>−/−</sup> BMMCs (Fig. 7, A and B). We next analyzed the response of BMMCs to SCF stimulation. Cells from wild-type and Lnk mutant mice showed a similar activation of Kit and phosphorylation of whole cell lysates (Fig. 7 C). To determine if a difference in the extent of MAPK activation might be responsible for the increased DNA synthesis rates observed in mutant cells (Fig. 6 C), we analyzed the duration of MAPK activation upon SCF stimulation. BMMCs from Lnk<sup>−/−</sup> mice displayed a higher level of phospho-ERK1/2 (1.5–4-fold), which decreased with a slower kinetics compared with wild-type BMMCs cells (Fig. 7, C and D). Together, these results suggest that Lnk negatively regulates signaling pathways downstream of both the IL-3Rβ and Kit receptors that are implicated in cell proliferation.

**Lnk Expression Is Regulated during Hematopoiesis.** Lnk is reportedly expressed throughout thymocyte development and at high levels in B220<sup>+</sup> splenic cells (20). However, the increased number of clonogenic progenitors observed in Lnk<sup>−/−</sup> mice suggests that Lnk expression may be regulated during hematopoietic differentiation. To assess Lnk expression during hematopoiesis, a hierarchy blot was hybridized with a radiolabeled Lnk cDNA probe including the terminal 3′ untranslated sequence (Fig. 8 A) and densitometric analysis was performed. Hybridization of the Lnk probe was strong in tripotent and unipotent precursors cells of megakaryocyte and myeloid lineages, as well as in mature megakaryocytes and macrophages, moderate in pentapotent and unipotent precursors cells of the erythroid lineage (BFU-E and CFU-E), and low in tetrapotent cells and other terminally mature cells (erythrocytes, neutrophils, mast, B and T cells).

To further characterize Lnk, we analyzed its expression in different organs. Lnk was highly expressed in some non-hematopoietic organs examined such as testis, brain, and muscle (Fig. 8 B). Interestingly, although Lnk expression is high in the bone marrow and lymph nodes, only low levels of Lnk RNA were detected in the spleen (Fig. 8 B), partially due to Lnk being weakly expressed in the mature cells of which the spleen is primarily composed. All slot blots were hybridized with a probe for L32, a constitutively expressed housekeeping transcript, to verify equal loading of all cDNAs (Fig. 8 C). The specificity of the Lnk probe was also confirmed by hybridization of a Northern blot. The same Lnk transcripts detected in wild-type and heterozygous cells with an NH<sub>2</sub>-terminal Lnk cDNA probe (Fig. 1 D) were observed with this 3′ Lnk probe (Fig. 8 C). These data demonstrate that Lnk is regulated in the hematopoietic compartment, and is most abundantly expressed in hematopoietic precursors.

**Discussion**

Lnk-deficient mice display an abnormal accumulation of erythroid cells, megakaryocytes, and B lymphocytes in the different hematopoietic compartments, indicating a defect in lymphoid and myeloid homeostasis. One of the most

---

**Figure 6.** Effect of Lnk disruption on cell proliferation in hematopoietic cells. Total bone marrow (A) or splenic cells (B) from wild-type and Lnk<sup>−/−</sup> mice were cultured with the indicated growth factors and proliferation was measured at day 4 (A) and day 6 (B) by an MTT assay. The values represent the mean (±SD) of triplicate determinations. (C) BMMCs from wild-type and Lnk<sup>−/−</sup> mice were cultured with the indicated concentrations of SCF or IL-3 for 28 h and proliferation was measured as [3H]thymidine incorporation. The values are the mean counts per minute (±SD) of triplicate determinations. All graphs are representative of two independent experiments, each done with two mice per genotype.
striking features of *Lnk*-deficient mice is a profound splenomegaly with excessive fibrosis and EMH. Morphologically, the abnormal expansion of B lymphocytes and erythrocytes causes considerable change in the architecture of the spleen, whereas the accumulation of a large number of megakaryocytes may be responsible for the extensive fibrosis observed. This phenotype resembles human myeloproliferative disorders that are characterized by abnormal proliferation of more than one hematopoietic cell lineage (49): the myeloid lineage in chronic myelogenous leukemia (CML); the erythrocytic and megakaryocytic lineages in polycythemia vera; and megakaryocytic cells in essential thrombocythemia and primary myelofibrosis. With the exception of CML, the molecular mechanisms responsible for the clonal proliferation in these disorders are unknown. Our results indicate that ablation of *Lnk* expression causes a dysregulation of hematopoiesis reminiscent of these diseases. *Lnk*-deficient mice exhibit amplification of both the erythroid lineage (specifically in spleen) and the megakaryocytic lineage, enhanced sensitivity to several cytokines, and abnormal responses to IL-3 and SCF. Interestingly, transgenic mice expressing activated forms of the IL-3Rβ chain display a chronic myeloproliferative disorder and an acute leukemia-like syndrome (50, 51). Moreover, it has been shown that the synergistic action of cytokines, such as SCF, IL-3, IL-7, and EPO, provides a growth advantage to multipotent progenitors and increases the frequency of myeloid, lymphoid, and erythrocytic progenitor cells (45, 52, 53). Indeed, preliminary analysis of erythroid progenitor colony growth in the presence of IL-3 and different concentrations of EPO, showed a hypersensitive pattern of growth in *Lnk*<sup>−/−</sup> cells (unpublished data). Therefore, it is possible that in the *Lnk* mutant mice, multiple cytokines are able to stimulate or continuously sensitize multipotent progenitors via synergistic combinations of growth factors, such as SCF, IL-3, and other cytokines.

One important question deals with the regulation of Lnk expression. This is particularly interesting because (1) Lnk expression is first detected in 11.5-d embryos, at a stage in which definitive hematopoiesis within the fetal liver is established (unpublished data; reference 54); (2) its expression is higher in hematopoietic precursors than in mature cells; (3) the *Lnk* mutant phenotype is already visible in newborn mice; and (4) *Lnk*<sup>−/−</sup> mice display a phenotype intermediate between wild-type and mutant mice. These data suggest that Lnk expression and function are tightly regulated.

Figure 7. Analysis of IL-3 and SCF signaling pathways in *Lnk*-deficient BMMCs. (A and B) BMMCs from wild-type (+/+) and *Lnk* mutant (−/−) mice were stimulated with 100 ng/ml IL-3 for 10 min or for the indicated times. IL-3Rβ chain was immunoprecipitated from wild-type and *Lnk*<sup>−/−</sup> BMMCs lysates and analyzed with anti-P-Tyr and anti–IL-3Rβ antibodies. Whole-cell lysates were subjected to Western blot analysis with anti–phospho-Akt and anti–phospho-ERK1/2 antibodies. Anti-ERK1/2 antibodies were used to show equal loading. Normalized densitometric analysis of ERK phosphorylation is shown in B. (solid line) Wild-type; (dashed line) *Lnk*<sup>−/−</sup>. (C and D) Total BMMCs lysates were stimulated with 100 ng/ml SCF for the indicated times and then subjected to Western blot analysis with anti-P-Tyr, anti-Kit, and anti–phospho-ERK1/2 antibodies. Anti-ERK1/2 antibodies were used to show similar loading. Normalized densitometric analysis of ERK phosphorylation is shown in D. (solid line) Wild-type; (dashed line) *Lnk*<sup>−/−</sup>. All data are representative of four independent experiments. Each experiment was done with cells isolated from two mice per genotype.
Our data with cells to undergo more rounds of proliferation or to survive associated with an increase in the number of cells within each cytokine sensitivity that would normally be controlled out of the bone marrow retain a proliferative capacity and

Figure 8. Lnk expression during hematopoiesis. (A and B) A slot blot containing either independent amplified cDNA samples from hematopoietic cells ranging from pentapotent precursors to terminally maturing cells in erythroid, myeloid, and lymphoid lineages (A) or different organs (B) was hybridized with a 3′ radiolabeled probe for Lnk. Radioactivity was quantified with a Phosphorimag (Bio-Rad Laboratories), and the results from A are shown in a hierarchy tree scheme: (black circles) high expression; (gray circles) moderate expression; (white circles) low expression. The ghosted circle represents a committed mast cell precursor stage, not actually sampled. E, erythroid; Meg, megakaryocyte; Mac or Mc, monocyte/macrophage; N, neutrophil; Mast or Mst, mast cell; B, B cells; T, T cells; BFU-E, precursors of large erythroid-only 6–8-d colonies; CFU-E, precursors of small erythroid 2-d colonies; p, precursors of 7-d colonies; pentapotent (E/Meg/Mac/N/Mst), tetrapotent (E/Meg/Mac/N), tripotent (E/Meg/Mac), and bipotent (E/Meg) precursor cells; 3T3, NIH3T3 fibroblasts; S17, stromal cell line; sc, stem cell; BM, bone marrow; LN, lymph node. (C) The same slot blots used in A and B were hybridized with the L32 probe to confirm equal loading. HC, hematopoietic cells; O, organs, same order as shown in A and B, respectively. A similar Northern blot as the one used in Fig. 1 D was hybridized with the 3′ Lnk probe used in A and B to confirm specificity.

during hematopoiesis. It is possible that the high levels of Lnk expression at later stages of hematopoiesis are important for attenuating cell proliferation and inducing the terminal stages of cell differentiation. This possibility is consistent with the phenotype of the Lnk-deficient mice, particularly the predominance of more immature cells in peripheral sites. In this regard, it was surprising to observe IL-7–responsive cells in the spleen of Lnk−/− animals. It is also possible that hematopoietic progenitors that migrate out of the bone marrow retain a proliferative capacity and cytokine sensitivity that would normally be controlled through the induction of Lnk expression. The increase in progenitor cell numbers from the Lnk mutant mice was associated with an increase in the number of cells within each colony. This increase could be due to the capacity of these cells to undergo more rounds of proliferation or to survive for longer. Our data with Lnk-deficient cells suggest that Lnk mainly exerts an effect on proliferation. In any case, it is possible that Lnk expression is increased before differentiation, thereby limiting colony size. Furthermore, it is evident that terminal differentiation can occur in Lnk mutant cells, demonstrating that Lnk is not essential for this function, but rather serves a regulatory role.

The phenotypes observed in Lnk-deficient mice exhibit several similarities with the phenotypes of mice deficient for other molecules involved in the regulation of hematopoietic signaling such as p62dok, SHIP, SHP-1, and SOCS3 (17, 55–57). For example, BMMCs derived from SHP-1−/− mice exhibit an augmented and prolonged activation of ERK-1 in response to SCF compared with that observed in Lnk−/− mice (58). These parallels suggest that Lnk may also function as a negative regulator of tyrosine kinase signaling in hematopoietic cells. The mechanism by which the Lnk family members influence growth factor and cytokine signaling is hypothesized to be through their ability to positively or negatively regulate the JAK/STAT and/or the Ras/MAPK pathways, based on overexpression studies of SH2-B and APS (27, 32, 34, 35). In this regard, we have not detected an effect of Lnk ablation on the activation of JAK2 in BMMCs stimulated with IL-3 (unpublished data). However, we have not yet examined the activation of the JAK/STAT pathway in other cytokine-stimulated progenitors. Experiments are in progress to analyze this pathway in more detail. Our results with Lnk−/− cells demonstrate that Lnk can modulate the kinetics of activation of Ras/MAPK, correlating with altered proliferation. Furthermore, Lnk appears important in modulating the cellular response to distinct growth factors (IL-3, IL-7, or SCF). A recent report has demonstrated that MAPK activation is required for the pBCR to signal proliferation of pre-B cells in low concentrations of IL-7 (39). It is possible that the elevated proliferation of Lnk−/− pre-B cells observed in these conditions (Fig. 5 A) is due to an inability to properly regulate ERK activation in the absence of Lnk expression. Identification of Lnk-interacting proteins may provide insight into how Lnk achieves its negative effect on cellular signaling, and preliminary data indicate that the p85 subunit of PI3K can associate with Lnk in B cells (unpublished data).

It was surprising to detect high levels of Lnk in nonhematopoietic tissues such as tests, brain, and muscle because the Lnk−/− phenotype appears to exclusively affect the hematopoietic system. This is likely explained by the fact that SH2B and APS are highly expressed in nonhematopoietic tissues and therefore could compensate for the absence of Lnk in other organs. APS is also highly expressed in the hematopoietic system, but mainly present in mature B cell lines (23, 31, 32). This suggests that APS functions in mature cells where Lnk expression is very low and therefore could explain the absence of a Lnk phenotype in terminally mature cells. Thus, it will be important to analyze the phenotypes of mice genetically deficient in other Lnk family members, separately or in combination, to establish the biological specificity of these adaptor proteins.

In conclusion, our results indicate that Lnk is an important regulator of cytokine signaling during hematopoiesis.
Because of its role in maintaining homeostasis, Lnk−/− mice provide a powerful model in which to pursue Lnk function in hematopoietic progenitor and mature cells. Future studies with cells derived from these mice should contribute to the understanding of the molecular mechanisms by which signaling thresholds control hematopoietic homeostasis.

We thank G. Caruana and R. Ingham for discussions and comments on this manuscript, S. Kulkarni for morula aggregation, K. Harpal and C. Wang for helping with the histology, J. Fawcett for the cryosections, and G. Gish for helping with the preparation of the Lnk antibodies.

This work was supported by grants from the Canadian Institutes of Health Research and the National Cancer Institute of Canada. T. Pawson is a distinguished Medical Research Council of Canada professor.

Submitted: 13 November 2001
Revised: 18 March 2002
Accepted: 30 April 2002

References

28. Kotani, K., P. Wilden, and T. Pillay. 1998. SH2-Bβ is an insulin-receptor adapter protein and substrate that interacts...