

Enhanced Hematopoiesis by Hematopoietic Progenitor Cells Lacking Intracellular Adaptor Protein, Lnk

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Abstract

Hematopoietic stem cells (HSCs) give rise to variety of hematopoietic cells via pluripotential progenitors and lineage-committed progenitors and are responsible for blood production throughout adult life. Amplification of HSCs or progenitors represents a potentially powerful approach to the treatment of various blood disorders and to applying gene therapy by bone marrow transplantation. Lnk is an adaptor protein regulating the production of B cells. Here we show that Lnk is also expressed in hematopoietic progenitors in bone marrow, and that in the absence of Lnk, the number and the hematopoietic ability of progenitors are significantly increased. Augmented growth signals through c-Kit partly contributed to the enhanced hematopoiesis by *lnk*^{-/-} cells. Lnk was phosphorylated by and associated with c-Kit, and selectively inhibited c-Kit-mediated proliferation by attenuating phosphorylation of Gab2 and activation of mitogen-activated protein kinase cascade. These observations indicate that Lnk plays critical roles in the expansion and function of early hematopoietic progenitors, and provide useful clues for the amplification of hematopoietic progenitor cells.

Key words: bone marrow transplantation • hematopoietic progenitors • c-Kit • stem cell factor • Gab2

Introduction

All blood cell lineages differentiate from hematopoietic stem cells (HSCs)* which self-renew to produce blood cells throughout the entire lifetime of the organism (1). Recent studies have demonstrated the potential of HSCs or progenitor cells in bone marrow to give rise not only to blood cells, but also to other cell types including hepatocytes, cardiac myocytes, and epithelial cells of the liver, lung, gastrointestinal tract, and skin (2–4). Amplification of HSCs or progenitors represents a potentially powerful approach to the treatment of various blood disorders, to the regeneration of damaged liver, heart, and lung tissues, and may also allow the use of gene therapy by bone marrow transplantation. A modest net gain in HSC numbers has been reported using serum-free cultures containing stem cell factor (SCF),

IL-11, and Flt-3 ligand (5). Thrombopoietin has also been shown to promote HSC survival and induce the proliferation of HSCs in the presence of SCF (6, 7). However, culture conditions that allow the amplification of HSCs while retaining their self-renewing abilities have not been established, and the mechanism for the self-renewal of HSCs remains poorly understood.

Lnk is an adaptor protein mainly expressed in lymphocytes (8–11). Lnk forms part of an adaptor protein family, together with APS and Src homology (SH)2-B, whose members share the presence of an NH₂-terminal homologous domain, followed by pleckstrin homology and SH2 domains, and a COOH-terminal conserved tyrosine phosphorylation site (11–13). Lnk regulates B cell production by negatively controlling the expansion of pro-B cells. We have previously reported that mutant mice lacking the *lnk* gene showed enhanced B cell production due to the hypersensitivity of B cell precursors to SCF, a ligand for c-Kit (11). The absence of Lnk confers upon immature bone marrow cells an enhanced ability to support B lymphopoiesis in adoptively transferred host animals, even in a competitive environment such as nonirradiated recombination activating gene (RAG)-2^{-/-} host (11).

The receptor tyrosine kinase c-Kit is a member of a subfamily that includes the platelet-derived growth factor

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*Abbreviations used in this paper: CFU-S, CFU in spleen; HSC, hematopoietic stem cell; Lin, lineage marker; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; SCF, stem cell factor; SH, Src homology; RAG, recombination activating gene.

(PDGF), CSF-1, and Flt3/Flk-2 receptors (14, 15). As with other receptor tyrosine kinases, stimulation of c-Kit by SCF binding leads to dimerization, transphosphorylation, and the recruitment and tyrosine phosphorylation of various cellular proteins, including phosphatidylinositol 3-kinase and Akt that rescue cells from apoptosis, and Gab2 that leads to the activation of mitogen-activated protein kinase (MAPK) pathways (16, 17). Through SCF binding, c-Kit is a key controlling receptor in a number of cell types including HSCs and hematopoietic progenitors, mast cells, melanocytes, and germ cells. The growth and survival of the stem cells for these cell types are severely affected by mutations in the gene loci for c-Kit (*Kit^W*) or SCF (*sl*) (15).

In this study, we show that Lnk is also expressed in hematopoietic progenitors, and that in the absence of Lnk, the number and the hematopoietic ability of progenitors in bone marrow are significantly enhanced. Augmented growth signals through c-Kit partly contribute to the enhanced hematopoiesis by *lnk^{-/-}* cells. Lnk is phosphorylated by and associates with c-Kit. Lnk selectively inhibits c-Kit-mediated proliferation by inhibiting tyrosine phosphorylation of Gab2 and activation of the MAPK cascade. These observations indicate that Lnk plays critical roles in the expansion and function of early hematopoietic progenitors, and provide useful clues in the understanding of the generation and proliferation of hematopoietic progenitor cells.

Materials and Methods

Mice. *lnk^{-/-}* mice used in this study were backcrossed with C57BL/6 (B6-Ly5.2) >10 times. *Kit^{W/+}* or *Kit^{Wv/+}* mice were purchased from The Jackson Laboratory. Mice congenic for the Ly5 locus (B6-Ly5.1) were bred and maintained at the animal facility of the Institute of Medical Science, University of Tokyo, Tokyo, Japan. All mice were housed and bled in specific pathogen-free conditions.

Flow Cytometry and RT-PCR. Single cell suspensions were prepared, and cells stained using predetermined optimal concentrations of the respective antibodies. The stained cells were then analyzed on a FACScan™ or FACScalibur™ instrument (Becton Dickinson). The following mAbs were used: FITC-conjugated anti-Scα-1 (E13-161.7); PE-conjugated anti-c-Kit (2B8); biotin-conjugated anti-TER-119; biotin-anti-Gr-1 (RB6-8C5); PE- or biotin-anti-CD3ε (145-2C11); FITC-anti-CD8 (53-6.7); PE-anti-CD4 (RM4-5); PE-anti-CD43 (S7) (all purchased from BD PharMingen); FITC- or biotin-anti-Mac-1 (M1/70); FITC-, PE-, or biotin-anti-B220 (RA3-6B2) (from Caltag); and biotin-anti-Ly5.2 (a gift of K. Ikuta, Kyoto University, Japan). PE-conjugated streptavidin (Ancell), or Tri-Color-conjugated streptavidin (Caltag) were used for biotin-coupled antibody staining. Bone marrow cells were depleted of lineage-committed cells using a MACS® system (Miltenyi Biotec) after incubation with a cocktail of biotin-conjugated antibodies against various lineage markers (Lins) (B220, CD3, Gr-1, Mac-1, and TER-119) and streptavidin-coupled microbeads. Resulting Lin⁻ cells were stained with PE-anti-c-Kit, and c-Kit⁺ or c-Kit⁻ cells sorted using a FACS Vantage™ (Becton Dickinson). Poly(A)⁺ RNA was isolated from purified cells using a Micro-Fast Track kit (Invitrogen), and first strand cDNA

templates synthesized by Superscript II reverse transcriptase (GIBCO BRL) using random primers (TaKaRa, Kyoto, Japan). Serial dilutions of cDNA templates were subjected to PCR amplification by using primer-sets encompassing several introns for Lnk (FWD primer: 5'-ATGCCTGACAACCTCTACAC, REV primer: 5'-ATTACACAGTCTGCCTCTCT) or β-actin (FWD primer: 5'-ACACTGTGCCCATCTACGAG, REV primer: 5'-CTAGAAGCACTTGCCTGCA). Cycling parameters were 1 min at 94°C, 2 min at 64°C and 3 min at 72°C for 34 cycles to detect *lnk* mRNA, or 27 cycles for β-actin. PCR products were separated through 1.0% agarose gels and stained with ethidium bromide.

Colony-forming Unit in Spleen Assay. Nucleated bone marrow cells (10⁵) prepared from *lnk^{+/+}* or *lnk^{-/-}* mice were injected into lethally irradiated (9.5 Gy) female wild-type mice. The recipient mice were killed 12 d after the injection, and their spleens were removed and fixed in Bouin's solution for macroscopic examination to count colonies.

Competitive Repopulation Assay. Bone marrow cells obtained from *lnk^{-/-}* or *lnk^{+/+}* mice (Ly5.2) were depleted of lineage-committed cells using a MACS® system as described above. Resulting Lin⁻ cells (7.0 × 10⁴) were intravenously injected into lethally irradiated (9.5 Gy) recipient mice together with 3.5 × 10⁵ competitor Lin⁻ cells obtained from B6-Ly5.1 mice. For assays using *lnk^{-/-}Kit^{+/+}* or *lnk^{-/-}Kit^{W/+}* mice, 1.5 × 10⁵ or 7.5 × 10⁴ bone marrow cells were injected into lethally irradiated recipient mice together with 1.5 × 10⁶ competitor bone marrow cells obtained from B6-Ly5.1 mice. Bone marrow cells, splenocytes, and thymocytes of chimeric animals were isolated and analyzed 10–12 wk after transfer.

Plasmid Construction and Transfection. Full-length *lnk* cDNA (11) was subcloned into the BamHI site of pcDNA3 (Stratagene), a eukaryotic expression vector driven by the cytomegalovirus enhancer and promoter, resulting in pcDNA3-Lnk. Substitutional mutations were introduced into the *lnk* cDNA sequence encoding the COOH-terminal tyrosine residue (Y536) by PCR-based site-directed mutagenesis. The introduced mutation was confirmed by DNA sequencing. The resulting mutated cDNA was subcloned into pcDNA3 (pcDNA3-Y536F). A mouse mast cell line, MC9 was grown in RPMI 1640 medium supplemented with 5% FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin and 10 U/ml mIL-3. MC9 cells were transfected by electroporation with pcDNA3-Lnk or pcDNA3-Y536F linearized with *Ssp*I. 2 d after transfection, cells were seeded into 96-well plates (10⁴ cells per well) and selected in medium containing 0.8 mg/ml G418. Transfectants resistant to G418 were expanded and screened for expression of Lnk protein by immunoblotting.

Proliferation Assay and Immunoblotting. Transfectants (10⁴) were cultivated in 200 μl of medium in 96-well plates. Cells were stimulated with various concentrations of SCF or IL-3. Cells were pulse-labeled with [³H]thymidine (0.2 μCi per well) during the last 16-h of the 72-h culture period, and incorporated [³H]thymidine measured using a MATRIX 96 Direct Beta Counter (Packard Instrument Co.). Transfectants were washed in Hanks' Balanced Salt solution three times and starved in RPMI 1640 medium containing 2% FCS, 50 μM 2-ME for 4 h. Cells were then collected and stimulated with 1 μg/ml SCF (Pepro-Tech) for the indicated times. After stimulation, cells were collected and lysed on ice in lysis buffer (150 mM NaCl, 50 mM Tris HCl, pH 7.5, 1% NP-40, 10 mM NaF, 1 mM Na₃VO₄, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and the lysates clarified by centrifugation. Whole

cell lysates or proteins immunoprecipitated using anti-Lnk (11) or anti-Gab2 (Upstate Biotechnology) were resolved by SDS/8%PAGE under reducing conditions, and transferred onto nitrocellulose membranes (Schleicher and Schull). After blocking with 5% skim milk/PBS, blots were probed with antiphosphotyrosine mAb (4G10; Upstate Biotechnology), anti-phospho-Akt, or anti-phospho-Erk1/2 (New England BioLabs), anti-c-Kit (Santa Cruz Biotechnology, Inc.) or anti-Lnk antibodies, and incubated with horseradish peroxidase-conjugated secondary antibodies. Filters were washed in 0.05% Tween 20, Tris-buffered saline, pH 7.5, and developed by enhanced chemiluminescence (NEN Life Science Products).

Results

Increased Number and Enhanced Ability of Hematopoietic Progenitors in *lnk*^{-/-} Mice. The hyperresponsivity of *lnk*^{-/-} precursor B cells to SCF and the enhanced ability to support B lymphopoiesis in nonirradiated RAG-2^{-/-} host animals (11) prompted us to investigate the function of Lnk in more immature hematopoietic progenitor cells. We first examined whether immature progenitors not yet committed to the B cell lineage expressed *lnk* mRNA. Bone marrow cells that already expressed Lins were depleted and resulting Lin⁻ immature cells

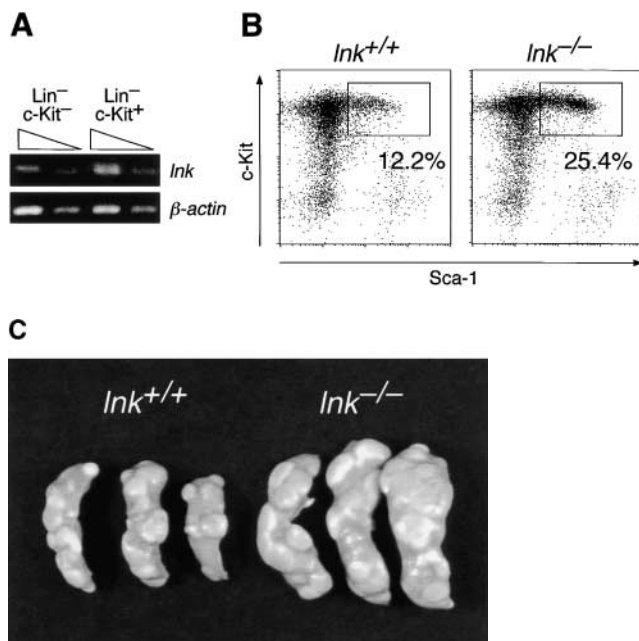


Figure 1. Increased hematopoietic progenitors in *lnk*^{-/-} mice. (A) Expression of *lnk* transcripts in hematopoietic progenitor cells of normal mice. Lin⁻ bone marrow cells were purified and separated according to c-Kit expression. Serial dilutions (threefold) of cDNA templates were prepared and subjected to RT-PCR analysis using primer sets designed to amplify *lnk* (top) or β -actin (bottom) cDNA fragments. (B) The cell fraction containing HSCs (Lin⁻Sca-1⁺c-Kit⁺) was increased in the bone marrow of *lnk*^{-/-} mice. Sca-1 and c-Kit expression on Lin⁻ bone marrow cells prepared from *lnk*^{+/+} or *lnk*^{-/-} mice was analyzed by flow cytometry. Representative dual parameter plots are shown. (C) Increased hematopoietic progenitors (CFU-S d12) caused by the *lnk*-deficiency. Bone marrow cells (10⁵) were transplanted into lethally irradiated recipient mice. The spleens were removed after 12 d and fixed in Bouin's solution to visualize the colonies.

were further purified by the expression of c-Kit. RT-PCR results demonstrated that *lnk* mRNA was expressed in Lin⁻ bone marrow cells. Among these Lin⁻ cells, more *lnk* transcripts were observed in immature c-Kit⁺ cells than in c-Kit⁻ cells (Fig. 1 A). Then we compared the distribution of cells expressing c-Kit and Sca-1 within the Lin⁻ cell population obtained from *lnk*^{-/-} or *lnk*^{+/+} mice. The Lin⁻c-Kit⁺Sca-1⁺ fraction, within which HSCs are found (18), was significantly expanded in *lnk*^{-/-} mice compared with *lnk*^{+/+} mice (Fig. 1 B, Table III). Pluripotential hematopoietic progenitors give rise to CFU in spleen (CFU-S) after 12 d when transplanted into lethally irradiated host. CFU-S d12 in *lnk*^{-/-} bone marrow was significantly increased compared with wild-type (Table I, Fig. 1 C). In addition, CFU-S d12 colonies derived from *lnk*^{-/-} cells were larger in size than those derived from *lnk*^{+/+} cells, suggesting the enhanced proliferating ability of *lnk*^{-/-} hematopoietic progenitors.

The ability of *lnk*^{-/-} progenitors to produce various hematopoietic cell types was examined by competitive repopulation assay. To avoid the contribution of increased B cell precursor populations in *lnk*^{-/-} bone marrow to recipient B cell compartments, Ly5.2⁺Lin⁻ cells were purified from *lnk*^{-/-} or *lnk*^{+/+} mice and transferred into irradiated hosts together with a fivefold excess of Ly5.1⁺ normal Lin⁻ cells (Fig. 2 A). Reflecting the ratio of testee to competitor cells, only small part of B220^{lo}CD43⁻ B-lineage cells in bone marrow and B220⁺ splenocytes were Ly5.2⁺ in recipient mice repopulated using *lnk*^{+/+} Ly5.2⁺ progenitors (Fig. 2 B). In contrast, when *lnk*^{-/-} Ly5.2⁺ progenitors were used, almost all of the B lineage cells were Ly5.2⁺ despite the original transfer of five times fewer *lnk*^{-/-} progenitors than normal Ly5.1⁺ competitor cells (Fig. 2 B). This also demonstrates the enhanced ability of *lnk*^{-/-} progenitors to produce B lineage cells compared with normal progenitors. Unexpectedly, results of the competitive repopulation assay showed that the *lnk*^{-/-} progenitors produced not only B lineage cells but also myeloid cells and T cells more efficiently than normal progenitors (Fig. 2 B, Table II). Most of the B220⁻CD43⁺ myeloid cells in bone marrow and Mac-1⁺ splenocytes in mice that received *lnk*^{-/-} progenitors were Ly5.2⁺. In addition, most CD4⁺CD8⁺ thymocytes and CD3⁺ splenocytes were generated from *lnk*^{-/-}

Table I. Increased CFU-S d12 in the Bone Marrow of the *lnk*^{-/-} Mice

Genotype	CFU-S d12 (per 10 ⁵ cells)
<i>lnk</i> ^{+/+}	11.5 ± 2.4
<i>lnk</i> ^{-/-}	20.8 ± 3.3 ^a

Bone marrow cells (10⁵) were administered intravenously to lethally irradiated recipient mice. The spleens were removed and fixed 12 d after injection, and the number of colonies was counted. The values are mean ± SD for quadruplicates.

^a*P* < 0.01.

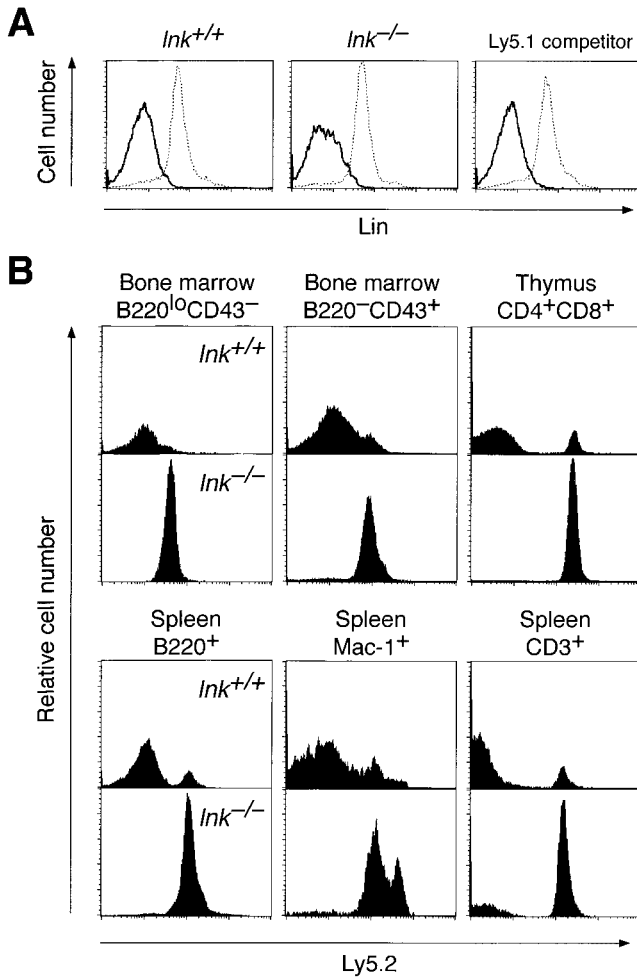


Figure 2. Enhanced repopulating ability of *lnk*^{-/-} progenitors demonstrated by a competitive repopulation assay. (A) Lin⁻ bone marrow cells prepared for repopulating assay. Bone marrow cells obtained from *lnk*^{-/-}, *lnk*^{+/+} mice, or B6-Ly5.1 (competitor) mice were depleted of lineage-committed cells using a MACS[®] system as described in Materials and Methods. Expression of Lin markers (B220, CD3, Gr-1, Mac-1, and TER-119) on cells before depletion (dotted lines) or after depletion (solid lines) was shown. (B) Lin⁻ cells obtained from *lnk*^{+/+} or *lnk*^{-/-} mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with a fivefold excess of Lin⁻ cells prepared from normal B6-Ly5.1 mice. Ly5.2 expression on B220^{lo}CD43⁻ B lineage cells in bone marrow, B220^{lo}CD43⁺ myeloid cells in bone marrow, CD4⁺CD8⁺ thymocytes, B220⁺ splenic B cells, Mac-1⁺ splenocytes, and CD3⁺ splenic T cells were analyzed 10–12 wk after transfer. Upper profiles show results obtained from animals transferred with *lnk*^{+/+} cells, and lower profiles show results obtained from animals transferred with *lnk*^{-/-} cells. Representative results of three independent experiments are shown.

progenitors even in the presence of five times more normal progenitors. Most of the IL-2R β ⁺CD3⁺ NKT cells in the spleen and thymus were also derived from *lnk*^{-/-} progenitors (data not shown). These results indicate that the ability of hematopoietic progenitors to generate various lineages of hematopoietic cells is greatly enhanced by the absence of Lnk.

c-Kit Signaling Is a Major Target Affected by *lnk* Deficiency. We have previously demonstrated that B cell overproduction observed in *lnk*^{-/-} mice was due, at least in part to

SCF hypersensitivity of the precursors, reflecting enhanced c-Kit signaling in the absence of Lnk (11). To confirm our observations in vivo, we bred *lnk*^{-/-} mice to *Kit*^{W/+} mutant mice. The *Kit*^W mutation consists of a deletion in the c-Kit protooncogene that results in reduced cell surface expression of c-Kit protein in *Kit*^{W/+} heterozygote mice (19). *Kit*^{W/Wv} mice are commonly used as viable mutant mice carrying nonfunctional c-Kit. However, the fraction of B lineage cells in the bone marrow of these mice varied from animal to animal, presumably due to malnutrition accompanied with severe anemia. Therefore, we compared *Kit*^{+/+} with *Kit*^{W/+} mutant mice in a *lnk*^{-/-} background. *Kit*^{W/+} mice (*lnk*^{+/+}*Kit*^{W/+}) showed no abnormalities in B cell development compared with normal (*lnk*^{+/+}*Kit*^{+/+}) mice (Table III). Total bone marrow preparations from *lnk*^{-/-}*Kit*^{+/+} mice contained higher cell numbers than from normal mice, with both B220^{lo}IgM⁻ and B220^{lo}IgM⁺ fractions significantly increased. Reduction of c-Kit expression by the introduction of the *Kit*^W mutation on a *lnk*^{-/-} background partially but significantly normalized B cell overproduction in *lnk*^{-/-}*Kit*^{W/+} mice (Table III). This confirms that the main reason for B cell overproduction in *lnk*^{-/-} mice is enhanced signaling through the c-Kit tyrosine kinase receptor.

The fraction of Lin⁻Sca-1⁺c-Kit⁺ progenitors in *lnk*^{-/-}*Kit*^{W/+} bone marrow was then examined. Expression levels of c-Kit were reduced in *lnk*^{-/-}*Kit*^{W/+} mice compared with *lnk*^{-/-}*Kit*^{+/+} mice (Fig. 3 A). Importantly, the increased Lin⁻Sca-1⁺c-Kit⁺ fraction in *lnk*^{-/-}*Kit*^{+/+} mice was partly but significantly reduced in *lnk*^{-/-}*Kit*^{W/+} mice (Fig. 3 A, Table III). This suggests that the increase in numbers of hematopoietic progenitors in *lnk*^{-/-} mice also results from enhanced c-Kit signaling. Despite the partial normalization of B cell overproduction and increased Lin⁻Sca-1⁺c-Kit⁺ progenitors, the competitive repopulation assay demonstrated an enhanced ability of hematopoietic progenitors from *lnk*^{-/-}*Kit*^{W/+} mice comparable to that from *lnk*^{-/-}*Kit*^{+/+} mice. Both *lnk*^{-/-}*Kit*^{W/+} and *lnk*^{-/-}*Kit*^{+/+} progenitors (Ly5.2⁺) generated most of the B and myeloid lineage cells and thymocytes in the presence of 10-fold more competitor cells (Ly5.1⁺) (Fig. 3 B, Table IV). Even in the presence of 20-fold more competitor cells, *lnk*^{-/-}*Kit*^{W/+} as well as *lnk*^{-/-}*Kit*^{+/+} progenitors continued to generate the majority of B, myeloid and T lineage cells (Fig. 3 B, Table IV). These results strongly suggest that the enhanced hematopoietic ability of *lnk*^{-/-} progenitors involves as yet unidentified mechanism(s), in addition to the enhanced c-Kit signaling that lead to the increase of Lin⁻ca-1⁺c-Kit⁺ progenitors.

Mechanisms for the Selective Inhibition of c-Kit Signaling by Lnk. The mechanism by which Lnk regulates signaling through c-Kit was studied using the SCF-dependent mast cell line MC9, which endogenously expresses low levels of Lnk (Fig. 4 A). MC9 transfectants that expressed Lnk at moderate (Lnk#1) or high (Lnk#2) levels were established and responsiveness to SCF examined. APS, a member of the Lnk adaptor family, is phosphorylated at a tyrosine residue at the COOH-terminal end and forms a binding site for c-Cbl (13), which is involved in the degradation of var-

Table II. Repopulating Ability of Hematopoietic Progenitors in Irradiated Hosts Is Enhanced by the Absence of *Lnk*

Experiment	Tested donor	Percent chimerism (Ly5.2 ⁺ cells, %)		
		Bone marrow B220 ^{lo} CD43 ⁻	Bone marrow B220 ⁻ CD43 ⁺	Thymus CD4 ⁺ CD8 ⁺
Exp. 1	Ly5.1 alone	2.3	7.4	0.1
	<i>lnk</i> ^{+/+}	10.9	28.9	0.1
	<i>lnk</i> ^{-/-}	87.1	48.9	99.8
	<i>lnk</i> ^{-/-}	85.1	78.1	87.5
Exp. 2	<i>lnk</i> ^{+/+}	16.6	23.9	17.2
	<i>lnk</i> ^{+/+}	18.0	16.9	16.1
	<i>lnk</i> ^{-/-}	97.5	93.9	99.2
	<i>lnk</i> ^{-/-}	97.6	95.8	99.4
Exp. 3	Ly5.1 alone	5.4	9.9	2.2
	<i>lnk</i> ^{+/+}	33.3	42.3	30.4
	<i>lnk</i> ^{-/-}	94.8	96.3	99.7
	<i>lnk</i> ^{-/-}	95.3	95.2	98.2

Lin⁻ bone marrow cells obtained from *lnk*^{+/+} or *lnk*^{-/-} mice (Ly 5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with a fivefold excess of Lin⁻ cells prepared from normal B6-Ly5.1 mice. Bone marrow cells and thymocytes were isolated 10–12 wk after transfer, and percentage of Ly5.2⁺ cells in the indicated cell fractions were determined.

ious proteins (20). The phosphorylation of the c-Cbl binding site is required for the negative regulatory functions of APS in cytokine receptor signaling, such as through PDGF-R and erythropoietin-R (21, 22). Therefore, we also generated transfectants that expressed a mutant Lnk (Y536F) that carried a substitution at the COOH-terminal tyrosine residue expected to be phosphorylated by analogy with APS. Lnk#2 and Y536F expressed c-Kit at amounts comparable with control transfectants (Neo), and c-Kit was similarly downregulated in all three transfectants upon stimulation with SCF (Fig. 4 B). Even when the transfectants were stimulated with low amounts of SCF, no differences between transfectants were detected (data not shown). This indicates that early signals are efficiently transmitted, and that the c-Kit degradation or internaliza-

tion is not affected in the presence of excess amounts of Lnk. Interestingly, SCF-induced proliferative responses were impaired in a dose-response manner by Lnk expression (Fig. 4 C). However, IL-3-induced proliferation remained intact. Y536F also showed impaired proliferation upon stimulation by SCF but not by IL-3. Apoptosis of MC9 cells induced by the depletion of SCF and IL-3 was not affected by overexpression of Lnk (data not shown). Therefore, Lnk selectively inhibits growth signaling mediated through c-Kit and the putative c-Cbl binding site of Lnk is not required for the negative regulatory effect on c-Kit.

We next analyzed the tyrosine phosphorylation of cellular proteins. SCF stimulation rapidly induced tyrosine phosphorylation of various cellular proteins, including c-Kit.

Table III. B Cell Overproduction and Increased in Lin⁻Sca-1⁺c-Kit⁺ Hematopoietic Progenitors in *lnk*^{-/-} Mice Is Partly Normalized by Reducing Expression of *c-Kit*

Genotype	Cell number (10 ⁷ cells/femur)	B220 ^{lo} IgM ⁻ (%)	B220 ^{lo} IgM ⁺ (%)		Lin ⁻ Sca-1 ⁺ c-Kit ⁺ (%)	(% in Lin ⁻ cells)	
<i>lnk</i> ^{+/+} <i>Kit</i> ^{+/+}	1.65 ± 0.16	19.5 ± 5.1	8.9 ± 3.6	(n = 12)	0.45 ± 0.07	16.2 ± 2.2	(n = 5)
<i>lnk</i> ^{+/+} <i>Kit</i> ^{W/+}	1.71 ± 0.19	18.2 ± 4.6	8.0 ± 3.1	(n = 10)	0.38 ± 0.06	14.9 ± 1.7	(n = 4)
<i>lnk</i> ^{-/-} <i>Kit</i> ^{+/+}	2.03 ± 0.37 ^a	3.6 ± 4.8 ^a	14.1 ± 2.1 ^a	(n = 11)	0.70 ± 0.06 ^a	26.1 ± 2.1 ^a	(n = 5)
<i>lnk</i> ^{-/-} <i>Kit</i> ^{W/+}	1.91 ± 0.28	28.9 ± 3.9 ^b	11.8 ± 1.7 ^b	(n = 8)	0.56 ± 0.13	21.3 ± 1.4 ^b	(n = 5)

Bone marrow cells obtained from 8–10-wk-old mice were analyzed, and mean cell number (±SD), mean percentage (±SD) of the indicated cell fractions and the number of mice analyzed are shown.

^aP < 0.05 compared to *lnk*^{+/+}*Kit*^{+/+}.

^bP < 0.05 compared to *lnk*^{-/-}*Kit*^{+/+}.

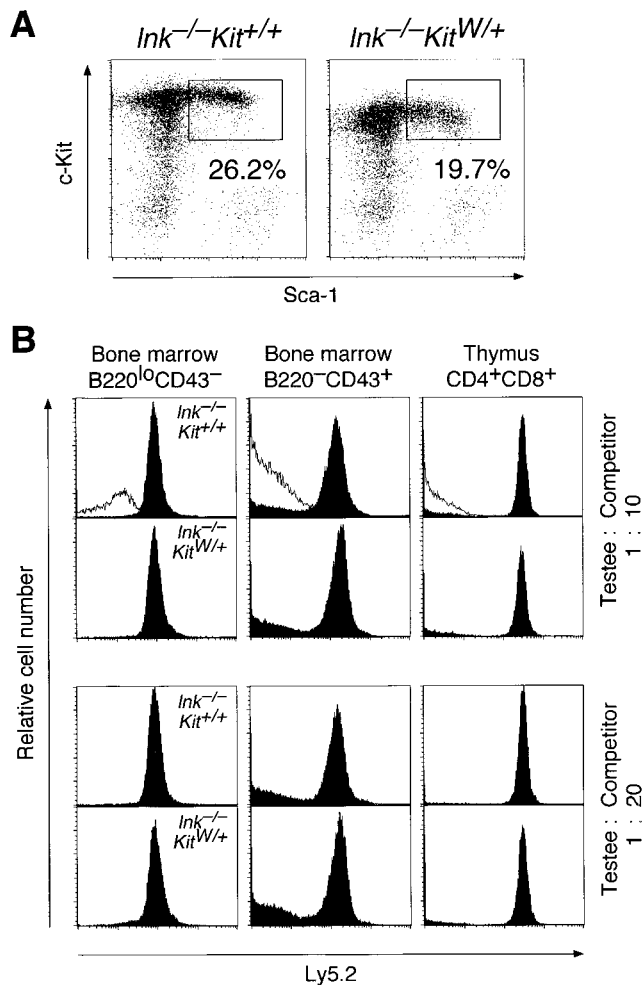


Figure 3. Increased number but not enhanced ability of *lnk*^{-/-} hematopoietic progenitors is partly normalized by reducing expression of c-Kit. (A) Expression of Sca-1 and c-Kit on Lin⁻ bone marrow cells of *lnk*^{-/-}*Kit*^{+/+} or *lnk*^{-/-}*Kit*^{W/+} mice. Shown are representative dual parameter plots analyzed by flow cytometry. (B) Comparable repopulating ability of progenitors obtained from *lnk*^{-/-}*Kit*^{+/+} or *lnk*^{-/-}*Kit*^{W/+} mice. Bone marrow cells obtained from *lnk*^{-/-}*Kit*^{+/+} or *lnk*^{-/-}*Kit*^{W/+} mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with 10 times (top) or 20 times (bottom) more competitor cells prepared from normal B6-Ly5.1 mice. Ly5.2 expression on B220^{lo}CD43⁻ B lineage cells in bone marrow, B220⁻CD43⁺ myeloid cells in bone marrow, and CD4⁺CD8⁺ thymocytes of chimeric animals were analyzed 10–12 wk after transfer. Upper profiles show results obtained from animals transferred with *lnk*^{-/-}*Kit*^{+/+} cells, and lower profiles show results obtained from animals transferred with *lnk*^{-/-}*Kit*^{W/+} cells. Thin lines in top panels show profiles of mice transferred with Ly5.1 competitor cells alone. Representative results of two independent experiments are shown.

Table IV. Comparable Repopulating Ability of HSCs Obtained from *lnk*^{-/-}*Kit*^{+/+} or *lnk*^{-/-}*Kit*^{W/+} Mice

Experiment	Tested donor	Testee: Competitor	Percent chimerism (Ly5.21 cells, %)		
			Bone marrow B220 ^{lo} CD43 ⁻	Bone marrow B220 ^{lo} CD43 ⁺	Thymus CD4 ⁺ CD8 ⁺
Exp. 1	Ly5.1 alone		4.7	5.1	0.3
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{+/+}	1:10	98.1	76.6	94.8
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{W/+}	1:10	98.4	73.1	77.5
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{+/+}	1:20	98.5	71.1	96.1
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{W/+}	1:20	94.4	64.4	24.6
Exp. 2	Ly5.1 alone		4.4	3.1	0.1
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{+/+}	1:10	81.2	65.8	81.2
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{W/+}	1:10	87.6	77.1	76.9
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{+/+}	1:20	86.1	67.3	97.0
	<i>lnk</i> ^{-/-} <i>Kit</i> ^{W/+}	1:20	87.2	74.5	86.8

Bone marrow cells obtained from *lnk*^{-/-}*Kit*^{+/+} or *lnk*^{-/-}*Kit*^{W/+} mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with 10-fold or 20-fold excess of competitor bone marrow cells prepared from normal B6-Ly5.1 mice. Bone marrow cells and thymocytes were isolated 10–12 wk after transfer, and percentage of Ly5.2⁺ cells in the indicated cell fractions was determined.

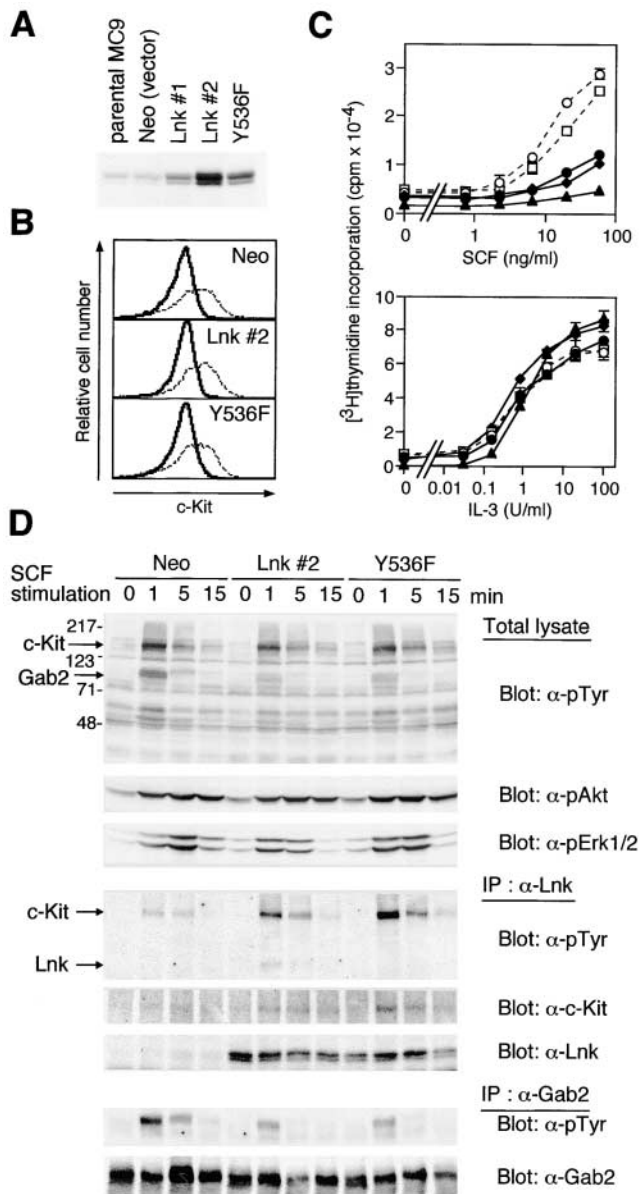


Figure 4. Lnk specifically inhibits c-Kit-mediated growth signaling in MC9 cells responsive to SCF or IL-3. (A) MC9 cells were transfected with expression plasmid without insert (Neo), plasmid encoding Lnk (Lnk#1, Lnk#2), or mutant Lnk (Y536F) that lacks the COOH-terminal tyrosine phosphorylation site. Lnk protein expression levels were analyzed by immunoblot. (B) Downregulation of c-Kit induced by SCF. Expression levels of c-Kit on unstimulated MC9 transfectants (broken lines) or cells stimulated with 100 ng/ml SCF for 30 min (solid lines) were analyzed by flow cytometry. Representative results of multiple experiments are shown. (C) Proliferative responses of parental MC9 (○), Neo (□), Lnk#1 (●), Lnk#2 (▲), and Y536F (◆) upon stimulation with various concentrations of SCF (top) or IL-3 (bottom). Values shown are mean \pm SD of triplicate determinations. Representative results of three independent experiments are shown. (D) Phosphorylation of cellular proteins induced by SCF stimulation of MC9 transfectants. Transfectants were starved for 4 h and stimulated with 1 μ g/ml of SCF for the indicated times. Whole cell lysates (top), anti-Lnk immunoprecipitants (middle), and anti-Gab2 immunoprecipitants (bottom) were separated through SDS/PAGE gels and transferred onto nitrocellulose membranes. Resultant membranes were probed with the indicated antibodies and visualized by ECL. Representative results of multiple experiments are shown.

The pattern of protein tyrosine phosphorylation was comparable between the three transfectants except for the attenuation of phosphorylation of a 90–100-kD protein in Lnk#2 and Y536F cells (Fig. 4 D). c-kit itself was efficiently phosphorylated, and we observed neither attenuated nor augmented c-Kit phosphorylation. Akt was also similarly phosphorylated among the three transfectants (Fig. 4 D). Interestingly, MAPK activation as detected by its phosphorylation was perturbed in Lnk#2 and Y536F, correlating with the attenuated phosphorylation of the 90–100-kD protein. Immunoprecipitation experiments demonstrated that Lnk was phosphorylated by and associated with c-Kit. This association was dependent on phosphorylation of c-Kit, but not of Lnk, as the mutant Lnk Y536F was no longer phosphorylated upon SCF stimulation but still efficiently bound c-Kit. Finally, the 90–100 kD phosphoprotein was identified as Gab2, which is thought to be a substrate of c-Kit (17). Anti-Gab2 reacted with and immunoprecipitated the 90–100 kD phosphoprotein (Fig. 4 D). These results demonstrate that Lnk is a substrate of c-Kit, specifically interacting with phosphorylated c-Kit, and that Lnk negatively regulates c-Kit-mediated signaling for cell growth, at least in part, by attenuating Gab2 phosphorylation and the subsequent activation of the MAPK pathway.

Discussion

We demonstrated that hematopoietic progenitors were increased in the bone marrow of *lnk*^{-/-} mice as a consequence of enhanced c-Kit signaling, and that *lnk*^{-/-} hematopoietic progenitors exhibited a greatly increased capacity to repopulate and produce various blood cell lineages after transplantation into lethally irradiated host animals. While it is likely that the increased progenitor fraction partly accounts for the enhanced hematopoiesis by *lnk*^{-/-} progenitors, the enhanced hematopoiesis by *lnk*^{-/-}*Kit*^{W/+} progenitors was comparable to that by *lnk*^{-/-}*Kit*^{+/+} progenitors in competitive repopulation assays. This suggests the existence of Lnk-dependent but c-Kit-independent signaling pathways that lead to the augmented ability of hematopoietic progenitors to repopulate host bone marrow and undergo hematopoiesis. Flt3/Flk-2 may be involved in such a pathway as it has been shown to support proliferation and differentiation of hematopoietic progenitor cells, while disruption of the Flt3/Flk-2 perturbed the production of various blood cell lineages (23). However, injection of anti-Flt3/Flk-2 antibodies into *lnk*^{-/-} mice failed to normalize the B cell overproduction in *lnk*^{-/-} mice (unpublished data), indicating that Flt3/Flk-2 signaling is not likely to be a target affected by the *lnk*-deficiency. Alternatively, the fact that the increased reconstituting activity of *lnk*^{-/-} progenitors was not affected by the reduction of c-Kit expression would indicate difference in the threshold of c-Kit-dependent signals in early hematopoietic progenitors or HSCs, late-stage progenitors and B cell precursors. Since c-Kit is not absent but only reduced in *Kit*^{W/+} mice, c-Kit signaling with a relatively low threshold in early progenitors or HSCs

might fully function in the condition where *c-Kit*-dependent growth signaling in the majority of *Lin*⁻*c-Kit*⁺*Sca-1*⁺ progenitors and B cell precursors were affected.

We found that Lnk is also expressed in hematopoietic progenitors and that the ability to generate various lineages of blood cells was significantly enhanced in irradiated host animals in the absence of Lnk. In *lnk*^{-/-} mice, however, the B lineage is affected to a greater degree while other lineages of cells are kept intact. The high expression of Lnk in B lineage cells (9) presumably accounts for the selective accumulation of B cells in *lnk*^{-/-} mice despite the importance of *c-Kit* in directing the development of a wide-range of blood cells. As Lnk expression is maintained at a high level in the B cell lineage in normal mice, B cell lineage is severely affected in the absence of Lnk. Although other lineage of *lnk*^{-/-} cells may also have some growth advantage compared with corresponding wild-type cells, the advantage is likely to be masked by a massively enhanced growth ability of B lineage cells in *lnk*^{-/-} bone marrow.

Biochemical analysis using *c-Kit*⁺ MC9 cells demonstrated that Lnk was tyrosine-phosphorylated by *c-Kit* and interacted with phosphorylated *c-Kit*. Lnk specifically inhibited *c-Kit*-mediated signaling for cell growth, at least in part, by attenuating Gab2 phosphorylation and the subsequent activation of MAPK pathway. Although the residue of Y536 of Lnk was the main target of *c-Kit*, tyrosine phosphorylation of Y536 was not required for the negative regulatory effect of Lnk on *c-Kit*. Moreover, SCF-induced downregulation of *c-Kit* was not affected by Lnk overexpression. These are in contrast to APS that inhibits Janus kinase (JAK)2 or PDGF-R-mediated signaling in combination with *c-Cbl*. Phosphorylation of the COOH-terminal tyrosine is essential for *c-Cbl* binding and subsequent inhibitory effects of APS (21, 22). The inhibitory function of Lnk on *c-Kit* is also in contrast to the positive regulatory roles of SH2-B and APS in signaling via receptors for various cytokines and growth factors (24–26), while both SH2-B and APS share significant similarities in structure with Lnk.

Phosphorylation of cellular proteins induced by *c-Kit* stimulation was hardly detectable in primary progenitor cells prepared freshly from normal or *lnk*^{-/-} bone marrow cells. Even if we used primary pro-B cells obtained by a long-term bone marrow culture, *c-Kit*-dependent phosphorylation was hardly detectable. Given the ubiquitous expression of MAPK pathway components and Gab2 (17, 27), however, the observation obtained using mast cell lines overexpressing Lnk should reflect the function of Lnk in hematopoietic progenitors exposed to low concentration of SCF in the bone marrow microenvironment. The transgenic mice overexpressing Lnk in B cell precursors resulted in the reduction of B lineage cells (unpublished data), presenting a good contrast to the phenotype of *lnk*^{-/-} mice and a reasonable synonym with the inefficient SCF-dependent growth of MC9 cells overexpressing Lnk. Recently, it has been reported that *Gab2*^{-/-} mice are viable and show normal steady-state hematopoiesis except reduced mast cells in skins and peritoneal cavity (28). *Gab2* is expressed

widely like its related protein Gab1 (17, 27), and both Gab1 and Gab2 are involved in MAPK activation in response to several growth factors and cytokines (17). Bone marrow-derived mast cells, however, express only Gab2 but not Gab1 (28). Although Lnk overexpression resulted in attenuated phosphorylation of Gab2 in mast cell line MC9, Lnk might also act on and regulate Gab1 in other lineages of cells. Whether Lnk regulates Gab1 as well as Gab2 and whether Gab1 and Gab2 function redundantly in hematopoietic progenitors remain to be elucidated.

The expression of *c-Kit* has been one of best markers for HSCs commonly used in various purification protocols to enrich HSCs (18, 29–32). The definitive hematopoiesis is severely perturbed by mutations in the gene loci for *c-Kit* (*Kit*^W) or SCF (*sl*) (15). In addition, administration of anti-*c-Kit* antibodies abrogates development of hematopoietic cells (29, 30). These indicate the importance of *c-Kit* expressed on HSCs. Recent report, however, has demonstrated the existence of *c-Kit*⁻ HSC fraction (33). Although *c-Kit*⁻ HSCs that are dormant/quiescent population do not radioprotect or form CFU-S, they show delayed or slow reconstitution kinetics when cotransplanted with radioprotective bone marrow cells. *c-Kit*⁻ HSCs can give rise to *c-Kit*⁺ HSCs not in primary recipients but in secondary recipients when transplanted repeatedly into irradiated hosts (33). One of possible dysregulations caused by the *lnk*-deficiency resulting in the enhanced hematopoiesis might be a rapid and augmented transition from *c-Kit*⁻ HSCs into *c-Kit*⁺ HSCs in irradiated hosts that is independent on *c-Kit* signaling.

Defined characterization of *lnk*^{-/-} HSCs by the evaluation of the hematopoietic ability of single HSC and the repopulation to various tissues other than blood cells will be of great interest. HSCs transferred into host animals need to migrate into bone marrow, find a marrow niche best suited for their self-renewal, then proliferate and differentiate. Studying the effect of the *lnk*-deficiency on HSCs and clarifying the processes affected by the *lnk*-deficiency would provide useful information to reveal HSCs function *in vivo*. In addition, identification of Lnk-dependent signaling pathways and target genes that exhibit altered expression levels in the absence of Lnk would provide useful clues in the understanding of the self-renewal abilities of HSCs or the expansion of progenitor cells. Inhibiting Lnk function by a mutant form of Lnk, inhibition of Lnk-dependent signaling pathways, or forced expression of Lnk-target genes may eventually allow us to control the expansion and generation of hematopoietic progenitor cells from HSCs.

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