Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways

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Erythropoietin (Epo), along with its receptor EpoR, is the principal regulator of red cell development. Upon Epo addition, the EpoR signaling through the Janus kinase 2 (JAK2) activates multiple pathways including Stat5, phosphoinositide-3 kinase (PI-3K)/Akt, and p42/44 mitogen-activated protein kinase (MAPK). The adaptor protein Lnk is implicated in cytokine receptor signaling. Here, we showed that Lnk-deficient mice have elevated numbers of erythroid progenitors, and that splenic erythroid colony-forming unit (CFU-e) progenitors are hypersensitive to Epo. Lnk-/- mice also exhibit superior recovery after erythropoietic stress. In addition, Lnk deficiency resulted in enhanced Epo-induced signaling pathways in splenic erythroid progenitors. Conversely, Lnk overexpression inhibits Epo-induced cell growth in 32D/EpoR cells. In primary culture of fetal liver cells, Lnk overexpression inhibits Epo-dependent erythroblast differentiation and induces apoptosis. Lnk blocks 3 major signaling pathways, Stat5, Akt, and MAPK, induced by Epo in primary erythroblasts. In addition, the Lnk Src homology 2 (SH2) domain is essential for its inhibitory function, whereas the conserved tyrosine near the C-terminus and the pleckstrin homology (PH) domain of Lnk are not critical. Furthermore, wild-type Lnk, but not the Lnk SH2 mutant, becomes tyrosine-phosphorylated following Epo administration and inhibits EpoR phosphorylation and JAK2 activation. Hence, Lnk, through its SH2 domain, negatively modulates EpoR signaling by attenuating JAK2 activation, and regulates Epo-mediated erythropoiesis. (Blood. 2005;105:4604-4612)

Introduction

Erythropoietin (Epo) is essential for red cell production by preventing committed erythroid progenitors from undergoing apoptosis and allowing them to proliferate and differentiate. Epo-/- and Epo receptor (EpoR)-/- mice die at embryonic day 13.5 (e13.5) due to severe fetal anemia.1 The EpoR belongs to the type I cytokine receptor family, characterized by a single transmembrane domain and a cytoplasmic tail lacking a kinase domain. A ligand-induced receptor homodimer conformational change leads to trans-phosphorylation and activation of Janus kinase 2 (JAK2).2,3 Erythroid development in JAK2-deficient embryos is arrested earlier and fetal anemia is more severe than that of EpoR-/- embryos, indicating JAK2 is essential for signaling downstream of multiple cytokine receptors, in addition to EpoR, that are important for primitive erythroid development.4,5

Activated JAK2 phosphorylates key tyrosine residues in the EpoR cytoplasmic domain, thereby providing docking sites for SH2 domain-containing downstream signaling molecules. EpoR activates signal transducers and activators of transcription 5 (Stat5), Ras/mitogen-activated protein kinase (MAPK), and phosphoinositide-3 kinase (PI-3K)/Akt pathways.2,3 These signaling modules have been implicated in hematopoiesis. Stat5 plays an important role in maintaining a high erythropoietic rate during fetal development and during stress responses in adult mice, as shown by studies using stat5a-/- stat5b-/- mice. Stat5ab-deficient erythroid progenitors exhibit high levels of apoptosis and are less sensitive to Epo.6 Moreover, the antiapoptotic role of Stat5 in EpoR signaling is mediated through its direct induction of Bcl-XL in erythroid cells.7

Intricate cytokine receptor signaling networks rely heavily on adaptor proteins. Lnk is a member of a newly discovered adaptor protein family. Lnk and other family members, APS and SH2-B, share a common protein organization. They do not possess a kinase domain but contain several protein-protein interaction domains: a proline-rich amino-terminus, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and a conserved tyrosine near the carboxyl-terminus.8

Among mice nullizygous for Lnk family members, Lnk-deficient mice show the most profound perturbation in hematopoiesis. Mice nullizygous for Lnk revealed an expansion of pro/pre and immature B cells.9 Moreover, overexpression of Lnk in aorta-gonad-mesonephros (AGM) primary cultures suppresses the emergence of CD45- hematopoietic cells, via inhibition of the stem cell factor (SCF)/c-Kit signaling pathways.10 We recently demonstrated that in both bone marrow and spleen, Lnk-deficient mice exhibit increased numbers of megakaryocytes with increased ploidy. Lnk-deficient megakaryocytes derived from bone marrow (BM) and spleen display enhanced sensitivity to thrombopoietin (Tpo) during in vitro culture. Lnk overexpression in BM progenitor...
cells attenuated Tpo-dependent megakaryocyte proliferation and endomitosis. Importantly, Lnk-deficient megakaryocytes show enhanced and prolonged activation of Tpo-induced signaling pathways, indicating Lnk is a physiologic negative regulator of Tpo-mediated signaling and megakaryocytogenesis. However, a role for Lnk in erythroid development or Epo/EpoR signaling has not been established.

Lnk-deficient mice have near-normal steady-state hematocrits, even though there are increased numbers of erythroid colony-forming unit (CFU-e) progenitors in the spleen. We hypothesized that any disturbance in erythropoiesis in Lnk-nullizygous mice might be drowned due to a large erythropoietic reserve capacity in adult animals. To test this, we challenged adult Lnk−/− mice with erythropoietic stress, and found that they showed superior recovery with an enhanced erythropoietic rate. Lnk-deficient splenic erythroid progenitor cells exhibited enhanced Epo activation of the Akt and MAPK signaling pathways. Importantly, Lnk-deficient CFU-e progenitors displayed enhanced sensitivity to Epo in forming erythroid colonies. To dissect the mechanism by which Lnk modulates Epo signaling, we found that overexpression of Lnk, but not the R364E mutant with a disrupted SH2 domain, inhibited Epo-dependent growth and signaling in 32D/EpoR cells. Extending this result using a novel primary culture of erythroid progenitors, we demonstrated that the Lnk SH2 domain was essential for blocking Epo-dependent erythroid differentiation and inducing erythroblast apoptosis. Furthermore, our results indicate that Lnk directly attenuates EpoR phosphorylation and JAK2 activation, thereby inhibiting major signaling pathways initiated by Epo/EpoR.

Materials and methods

Mice, cell lines, and cytokines
Lnk-deficient mice were generously provided by Dr Tony Pawson (Samuel Lunenfeld Research Institute, Toronto, Canada), and have been backcrossed to the C57Bl/6 background for 7 generations. Epo was generously provided by Amgen (Thousand Oaks, CA). Interleukin 3 (IL-3)–dependent 32D cells were made as described. The Lnk constructs in the MSCV-IRES-GFP (MIG) vector were digested with BamH I and EcoR I sites and ligated to the MSCV-IRES-hCD4 (MIDC4) vector linearized by BgII and EcoRI. The Lnk constructs in the MSCV-IRES-GFP (MIG) vector were made as described.

Retroviral constructs
pcDNA-Lnk was generously provided by Dr Satoshi Takaki (University of Tokyo, Japan). Lnk cDNA was digested with BamH I and EcoRI sites and ligated to the MSCV-IRES-hCD4 (MIDC4) vector linearized by BgII and EcoRI. The Lnk constructs in the MSCV-IRES-GFP (MIG) vector were made as described.

Fetal liver cell preparation and culture
Fetal liver cells were isolated from e13.5 or e14.5 Balb/c mice (Jackson Laboratories, Bar Harbor, ME), and Ter119+ progenitor-rich erythroid cells were purified as described earlier. Cells were then plated at 1 × 10^5/mL to 2 × 10^7/mL in a 12-well plate and spin-infected for one hour with the desired retroviral supernatant. Transduced cells were then cultured in Iscoves modified Dulbecco medium (IMDM) containing 15% fetal bovine serum (FBS), 30 ng/mL SCF, 20 ng/mL Flt3L, and 10 ng/mL IL-6 for 6 hours, to allow expression of transduced genes and maintenance of the progenitor state. The cells were subsequently washed and resuspended in 1 mL media containing 2 U/mL Epo on fibronectin-coated plates. Some experiments included controls where Epo was omitted. On the second day, the media were replaced by 2 mL erythroid differentiation media (EDM) as described elsewhere.

Cytology
Cultured fetal liver cells were washed and resuspended in phosphate-buffered saline (PBS) with 2% FBS at a concentration of 3 × 10^6/mL. Cells (100 μL per slide) were centrifuged onto slides at 1000 rpm for 5 minutes (Cytospin 3; Thermo Shandon, Pittsburgh, PA). The slides were air dried and stained with 3,3’ diaminobenzidine (Sigma, St Louis, MO) and Wright-Giemsa (Sigma) according to the manufacturer’s recommendation. Cytology images were taken using an Olympus BH-2 microscope (Olympus, Melville, NY) with Splan FL2 100× objective lenses and a C-35 AD-2 camera. Numerical aperture was 1.25 Oil. Images were then scanned using Nikon Super Coolscan 4000ED (Nikon, Melville, NY) and acquired with Adobe Photoshop software (Adobe Systems, San Jose, CA).

Immunostaining and flow cytometry analysis
One day or 2 days after the culture of retroviral-transduced erythroid progenitor cells, the resulting erythroblasts were removed from the culture plates. Cells were then stained for hCD4, Ter119, and CD71 as described earlier. For apoptosis analysis, the erythroblasts were stained first for hCD4, then incubated with phycoerythrin (PE)–conjugated annexin V and 7-AAD (7-actinomycin D; BD Pharmingen, San Diego, CA) for 15 minutes at room temperature according to the manufacturer’s protocol. Fluorescence activated cell sorting (FACS) analysis was carried out using a Becton Dickinson FACSCalibur.

Colony assays
To detect CFU-e colonies, 1 × 10^5 BM and 1 × 10^6 spleen cells from wild-type mice, and 1 × 10^5 BM and 5 × 10^5 spleen cells from Lnk−/− mice were plated in duplicate in semisolid methylcellulose (M3334; StemCell Technologies, Vancouver, BC, Canada) according to the manufacturer’s protocol. Benzidine-positive (Sigma) colonies were counted after 2 days in culture. To detect erythroid burst-forming unit (BFU-e) colonies, 1.5 × 10^6 BM and 3 × 10^5 spleen cells from wild-type mice and 7.5 × 10^5 BM and 1 × 10^5 spleen cells from Lnk−/− mice were plated in duplicate in methylcellulose (M3434; StemCell Technologies) according to the manufacturer’s protocol. The BFU-e colonies were counted after 8 to 10 days in culture. To test Epo sensitivity of CFU-e progenitors, cells from wild-type and Lnk-deficient spleens were isolated and plated in duplicate in methylcellulose (M3234; StemCell Technologies) containing varying concentrations of Epo. Same numbers of cells were plated as described above.

Erythropoietic stress test
Wild-type and Lnk-nullizygous mice (7 to 9 weeks old; 22 g to 28 g per mouse) were injected intraperitoneally with phenylhydrazine (PHZ) at 60 mg/g body weight on days 0 and 1. Mice of both genotypes were divided into 2 subgroups, and retro-orbital vein bleeds were obtained from each subgroup at alternative intervals, in order to prevent phlebotomy-induced anemia. Hematocrits were measured on a hematocrit centrifuge (Micro-MB; International Equipment, Needham, MA). Freshly drawn blood was stained with new methylene blue “N” (J.T. Baker, Phillipsburg, NJ) for detecting RNA in reticulocytes. Reticulocyte count was subsequently measured by counting the portion of reticulocytes in total red cells on blood smears. The corrected reticulocyte count was calculated as described in the legend to Figure 1.

Protein lysates and Western blot analysis
After sorting 32D/EpoR cells for the green fluorescent protein–positive (GFP+) population expressing either vector alone or a vector encoding Lnk, we starved the cells in RPMI containing 1% bovine serum albumin (BSA) and subsequently stimulated them with 10 U/mL Epo for 0, 10, 30, and 120 minutes. The cell lysates were Western blotted with the indicated antibodies: anti-Stat5 (1:500, C-17; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p-Stat5 (pTyr 694), p-MAPK (pThr202/Tyr204), and p42/44 MAPK antibodies (1:1000; Cell Signaling Technology, Beverly, MA).

Ter119+ erythroid progenitor cells were transduced and cultured as described in “Fetal liver cell preparation and culture,” or directly cultured in...
Lnk-deficient mice were used and pooled in each experiment. Shown here are representatives of 3 independent experiments, and 3 wild-type and 3 Lnk-deficient mice were injected with phenylhydrazine (PHZ) at days 0 and 1 (indicated by arrows). Hematocrits (A) and reticulocyte counts (B) were measured at indicated intervals (mean ± standard deviation [SD]). The corrected reticulocyte count (also known as reticulocyte index) was calculated assuming a normal hematocrit of 45%, as follows: corrected reticulocyte count (%) = reticulocyte count (%) × (hematocrit / 45). There were 8 to 9 mice used in 2 independent studies. Student’s t tests were performed and P values are shown on top of each pair of data points. (C) At days 4 and 5 after PHZ treatment, spleen cells were isolated. CD71+ populations were purified using magnetic beads, and stimulated with 0, 1, 10, or 100 U/mL Epo for 10 minutes. Protein lysates from equal cell numbers were subjected to Western blotting analysis. Phosphorylated and total protein levels of p42/44MAPK and Akt are shown. Results shown here are representatives of 3 independent experiments, and 3 wild-type and 3 Lnk-deficient mice were used and pooled in each experiment.

Enhanced erythropoietic recovery and Epo/EpoR signaling in Lnk-deficient mice

We found that Lnk-deficient mice displayed increased erythroid progenitor numbers. As shown in Table 1, while the numbers of CFU-e progenitors showed only slight differences in Lnk-deficient BM, BFU-e progenitor numbers (per 2 × 10^5 BM cells) increased 1.7-fold compared with wild-type controls (Table 1, column 4). Taking into account that there are 30% more BM cells in Lnk-deficient mice relative to normal mice (Table 1, column 4), the total number of BFU-e progenitors in Lnk-deficient BM is 2.2-fold greater than in wild-type mice (Table 1, column 5). Spleens from Lnk-deficient animals displayed more profound differences than did BM. Splenic BFU-e and CFU-e progenitors increased 1.7- and 2.4-fold in frequency respectively, compared with wild-type mice, when equal numbers of cells were analyzed (Table 1, column 4). Since there are twice (1.9 ×) as many spleen cells in Lnk-deficient mice relative to wild-type mice, the total numbers of BFU-e and CFU-e progenitors are increased 3.2- and 4.6-fold, respectively, in Lnk-deficient spleen (Table 1, column 5). Thus, Lnk deficiency resulted in an abnormal increase in erythroid progenitor numbers in both BM and spleen.

Even though Lnk-deficient mice have increased numbers of erythroid progenitors, they exhibit near-normal steady-state hematocrits (Figure 1A and Velazquez et al12). Since adult mice have a large erythropoietic reserve capacity, quantitative differences in erythropoietic rate might be masked at the steady state. We therefore tested Lnk nullizygous mice for their ability to generate high erythropoietic rates under stress. We challenged the mice with a chemical-induced hemolytic anemia by phenylhydrazine (PHZ) injection at days 0 and 1 (Figure 1A). The hematocrits of mice from both groups dropped sharply to 26% at day 2 (Figure 1A). Wild-type mice continued decreasing their hematocrits, reaching a nadir (22%) at day 4 (Figure 1A). In contrast, Lnk-deficient mice quickly recovered their hematocrits to 32% by day 4. At day 6, Lnk-deficient mice still exhibited significantly higher hematocrits than those of wild-type mice (Figure 1A). By days 8 to 11, both wild-type and Lnk-deficient mice recovered their normal hematocrits (Figure 1A).

Reticulocytes are red cells newly generated from bone marrow and are prematurely released into the circulation under stress.

Table 1. Erythroid progenitor numbers from the BM and spleens of wild-type and Lnk nullizygous mice

<table>
<thead>
<tr>
<th>Progenitors by tissue type</th>
<th>Wild type*</th>
<th>Lnk−/−</th>
<th>Fold increase (P)†</th>
<th>Fold increase per mouse</th>
</tr>
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<tbody>
<tr>
<td><strong>BM</strong></td>
<td></td>
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<tr>
<td>Total cells per mouse, × 10^6</td>
<td>50 ± 12</td>
<td>63 ± 10</td>
<td>1.3 (.03)</td>
<td>1.3</td>
</tr>
<tr>
<td>BFU-e per 2 × 10^5 cells</td>
<td>10 ± 4</td>
<td>17 ± 5</td>
<td>1.7 (.01)</td>
<td>2.2</td>
</tr>
<tr>
<td>CFU-e per 2 × 10^5 cells</td>
<td>593 ± 100</td>
<td>517 ± 80</td>
<td>0.9 (.04)</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Total cells per mouse, × 10^6</td>
<td>150 ± 13</td>
<td>286 ± 68</td>
<td>1.9 (&lt;.001)</td>
<td>1.9</td>
</tr>
<tr>
<td>BFU-e per 2 × 10^5 cells</td>
<td>30 ± 11</td>
<td>52 ± 18</td>
<td>1.7 (.03)</td>
<td>3.2</td>
</tr>
<tr>
<td>CFU-e per 1 × 10^5 cells</td>
<td>230 ± 45</td>
<td>546 ± 35</td>
<td>2.4 (.01)</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Mice (8 to 12 weeks old) were used to isolate BM and spleen cells. Total BM and spleen cell numbers (mean ± SD) were enumerated in the presence of 3% acetic acid using a hemocytometer. An equal number of cells was plated in methylcellulose culture and enumerated for 2 days (CFU-e) and 10 days (BFU-e) after plating. The data were obtained from 6 to 8 mice from each genotype. Fold increase = Lnk−/−/wild type. Fold increase per mouse (fifth column) = fold increase (fourth column) × fold increase in total cells.

*Data shown are mean plus or minus SD.
†P values determined by Student’s t test.
Therefore, the proportion of reticulocytes in the peripheral blood red cell population is indicative of the erythropoietic rate. The basal reticulocyte count is less than 2% (Figure 1B). Under erythropoietic stress, the reticulocyte count in wild-type mice reached a maximal level of 19% at day 4 (Figure 1B). This rapid increase in reticulocyte count was more pronounced in Lnk-deficient mice, reaching a maximal level of 31% at day 4 (Figure 1B). In summary, Lnk nullizygous mice exhibited a less severe PHZ-induced anemia and a faster recovery from erythropoietic stress.

At days 4 to 7 after PHZ treatment, spleens are enriched for Epo-responsive erythroid progenitors, and these progenitor cells are mostly CD71+. We purified CD71+ splenic erythroid progenitor cells and stimulated them with different concentrations of Epo (Figure 1C). With increasing concentrations of Epo (0 U/mL to 100 U/mL), wild-type CD71+ erythroblasts showed increasing activation of Akt and p42/44MAPK (Figure 1C), with maximal activation of both signaling pathways at 10 U/mL to 100 U/mL Epo. Strikingly, Lnk-deficient CD71+ erythroblasts exhibited higher activation of Akt and MAPK at all Epo concentrations (Figure 1C). Thus, the absence of Lnk results in enhanced signaling pathways induced by Epo.

### Lnk-deficient CFU-e progenitors show enhanced sensitivity to Epo

To determine whether Lnk deficiency results in increased Epo sensitivity at the cellular level, erythroid progenitors from either wild-type or Lnk−/− mice were exposed to a range of Epo concentrations, and erythroid colony formation was monitored. Figure 2 shows that Lnk-deficient CFU-e progenitors were indeed more responsive to lower concentrations of Epo than wild-type controls. Interestingly, BM CFU-e progenitors from these mice showed similar sensitivity to Epo (data not shown).

### The Lnk SH2 domain is required for inhibiting Epo-dependent growth and signaling pathways in 32D/EpoR cells

To study the mechanism by which Lnk inhibits Epo/EpoR signaling pathways, we overexpressed Lnk in IL-3–dependent 32D hematopoietic cells. To this end, we first established a stable cell line expressing the EpoR, designated as 32D/EpoR cells. The effect of Lnk in Epo-dependent 32D cell growth was then examined by retroviral-transduction of wild-type Lnk using a bicistronic MSCV-IRES-GFP (MIG) vector. As GFP expression is tightly correlated with the expression of the gene cloned upstream of the internal ribosome entry site (IRES), we were able to identify cells expressing Lnk by analyzing GFP fluorescence. We introduced either vector alone or Lnk into 32D/EpoR cells and determined the fraction of GFP+–infected cells 2 days later. As shown in Figure 3A, while Lnk expressing 32D/EpoR cells declined in number during the 4-day experiment period, control cells exhibited exponential growth.

In order to investigate the role of different domains of Lnk in growth inhibition, we generated point mutations in the Lnk cDNA to ablate individual domains and overexpressed these mutant forms of Lnk in 32D/EpoR cells (Figure 3B). We determined the initial fraction of GFP+–infected cells 2 days after infection. We then cultured the cells in Epo and measured the GFP+ fraction every 3 days thereafter. The percentage of GFP+ cells relative to that at 2 days after infection was plotted. Results shown are representative of more than 5 independent experiments. (C-D) 32D/EpoR cells infected with either vector alone or the wild-type or the mutant forms of Lnk were purified, stimulated with Epo, and lysed at indicated intervals followed by Western blotting analysis. (C) Stat5 phosphorylation and protein levels after Epo administration. (D) P42/44 MAPK phosphorylation and protein levels after Epo administration. Representatives of 3 independent experiments are shown.
level of Stat5 phosphorylation dropped at 120 minutes (Figure 3C, lanes 1-4). Overexpression of Lnk abolished Stat5 activation induced by Epo (Figure 3C, lanes 5-8), but total Stat5 protein levels were unchanged (Figure 3C, lower panel). Similarly, in control cells, Epo induced maximal p44/42 MAPK phosphorylation at 10 minutes, and the level of MAPK phosphorylation dropped thereafter (Figure 3D, lanes 1-4). Lnk overexpression diminished this induction (Figure 3D, lanes 5-8). Total MAPK protein levels were unaffected (Figure 3D, lower panel).

Figure 3C-D also shows that the Lnk SH2 domain is essential for the inhibition of Stat5 and MAPK activation by Lnk. Lnk (R364E)–transduced cells showed similar Stat5 and MAPK activation to control cells (Figure 3C-D, lanes 9-12). In contrast, Lnk (W191A)– and Lnk (Y536F)–expressing cells attenuated the activation of Stat5 and MAPK to an extent similar to that by wild-type Lnk (Figure 3C-D, lanes 13-20). Thus, the Lnk SH2 domain is essential for inhibiting EpoR signaling and Epo-dependent cell growth in 32D cells, whereas neither the conserved tyrosine at the C-terminus nor the PH domain is required for these inhibitory functions.

The Lnk SH2 domain is required to inhibit Epo-dependent fetal liver erythroblast survival

In order to investigate whether Lnk affects Epo function in red cell development, we examined primary fetal liver erythroid progenitors. Ter119– erythroid progenitor cells were purified from e13.5 or e14.5 fetal livers, and retrovirally transduced with either vector alone or Lnk. Ter119– progenitor cells, when cultured in the presence of Epo, divide 4 to 5 times, and differentiate into enucleated reticulocytes, a process remarkably similar to that seen in vivo. Since infection rates routinely reach 90%, no further purification of infected cells is necessary.

We first quantitated the effect of Lnk on Epo-dependent primary erythroblast expansion (Figure 4A). By the end of a 2-day culture in the presence of Epo, control cells increased about 15-fold in number, whereas control cells cultured in the absence of Epo increased only 4-fold (Figure 4A). Lnk expressing erythroblasts cultured in the presence of Epo also increased only 4-fold, similar to control cells cultured in the absence of Epo (Figure 4A).

We next analyzed the mechanism responsible for Lnk-mediated inhibition of Epo-dependent cell expansion. At the end of a 2-day culture, those infected with the control vector contained 6% apoptotic (annexin V+ and 7-AAD−) and 6% dead cells (annexin V+ and 7-AAD+; Figure 4B). Overexpression of Lnk resulted in 33% apoptotic and 25% dead cells, numbers similar to those of control cells cultured in the absence of Epo (30% apoptotic and 21% dead cells; Figure 4B). However, Lnk did not block cell cycle progression of proerythroblasts after 16 to 18 hours of culture in the presence of Epo. Control cells exhibited exponential growth with 31%/57%/12% G1/S/G2 populations, which is similar to the cell cycle progression of Lnk-expressing cells, 33%/59%/8% G1/S/G2 populations. Interestingly, the lack of Epo also did not block cell cycle progression; control cells grown in the absence of Epo showed 34%/56%/10% G1/S/G2 populations. Thus, the cell number reduction in Lnk-expressing cells is due to increased apoptosis.

Consistent with the results obtained from 32D cells, the Lnk SH2 mutant R364E did not affect erythroblast survival when overexpressed in Ter119– erythroid progenitor cells (Figure 4). In contrast, the PH domain mutation (W191A) or Y536F mutation moderately disrupted Lnk inhibitory function (Figure 4). Thus, the Lnk SH2 domain is crucial for, and the PH domain and the conserved tyrosine contribute to, its inhibitory function in Epo-mediated erythroid cell survival.

The Lnk SH2 domain is required to inhibit Epo-dependent fetal liver erythroblast differentiation

We next examined whether Lnk affects erythroid differentiation in the primary erythroid culture system as described earlier. Cultured erythroblasts were stained for erythroid-specific marker Ter119 and nonerythroid-specific transferrin receptor CD71. Five distinct populations can be defined by their characteristic staining patterns: Ter119lowCD71med (primitive progenitor cells and proerythroid cells, Figure 5A, R1); Ter119lowCD71high (proerythroblasts and early basophilic erythroblasts, Figure 5A, R2); Ter119highCD71high (basophilic erythroblasts, Figure 5A, R3); Ter119highCD71med (chromatophilic and orthochromatophilic erythroblasts, Figure 5A, R4), and Ter119highCD71low (late orthochromatophilic erythroblasts and reticulocytes, Figure 5A, R5). Therefore, these cell populations correspond to progressive developmental stages, with R1 being the least and R5 being the most differentiated erythroid cells.

In freshly isolated e14.5 fetal liver cells, about 15% are Ter119– (Figure 5A). After magnetic bead purification, Ter119– populations are routinely enriched to about 95% with most of them being early erythroid progenitor cells (R1 and R2, Figure 5B). After 16 to 18 hours of culture of Ter119– progenitors in the presence of Epo, control erythroblasts (R1 and R2 cells) differentiate into R2 and R3 cells, shown as CD71high and Ter119low to high (Figure 5D, left
panels). Following a second day of culture, the erythroblasts further differentiate into benzidine-positive R3-R5 cells (Figure 5D, right panels).

In contrast, control erythroblasts cultured in the absence of Epo did not show hyper-upregulation of CD71/TfR (Figure 5E, left panels); they showed only 50% of the expression levels of CD71/TfR relative to control cells cultured in the presence of Epo for 16 to 18 hours of culture. Dashed line outlined regions (R0) indicate nonerythroid cells. The FACS plots were gated for the hCD4^+ population, that is, for expression of the transduced genes, in all the plots. For cytology the cells were centrifuged onto slides and stained with benzidine (brown) and Giemsa (purple for the nuclei). The arrowheads indicate benzidine-positive enucleated reticulocytes, and the thin arrows point to nonerythroid cells. Scale bars are 20 μm.

In summary, the Lnk SH2 domain is essential to block Epo-dependent erythroid differentiation by preventing CD71/TfR upregulation.
The SH2 domain of Lnk is required to inhibit Epo-induced Stat5, Akt, and MAPK activation in primary erythroid cells

To investigate the mechanism by which Lnk inhibits Epo-dependent erythroblast survival, we examined Epo-induced signaling pathways in primary fetal liver erythroid cells transduced with either the control vector or the vector encoding Lnk. Epo stimulated maximal phosphorylation of p44/42MAPK, Akt, and Stat5 in 10 minutes in control erythroblasts (Figure 6A-C, top lanes). Lnk overexpression attenuated the activation of p44/42MAPK and Akt, and abolished the activation of Stat5 (Figure 6, top lanes). In addition, the Lnk SH2 mutant (R364E) that did not affect erythroblast survival, did not affect Epo-induced MAPK, Akt, and Stat5 activation in primary erythroblasts (Figure 6, top lanes). In contrast, erythroblasts expressing Lnk (W191A) or Lnk (Y536F) showed similar blunted activations of Epo-induced signaling pathways to that of erythroblasts expressing wild-type Lnk (Figure 6, top lanes). The total protein levels remained unchanged among all the samples (Figure 6A, middle panel; 6B-C, bottom panels) and Lnk expression levels also were similar in cells expressing the different Lnk mutants (Figure 6A, bottom panel). The SH2 domain of Lnk is therefore crucial for Lnk’s inhibitory function in Epo-mediated signaling pathways in primary erythroblasts; whereas the PH domain and the conserved C-terminal tyrosine (Y536) are not.

The Lnk SH2 domain is required to inhibit Epo-induced JAK2 activation and EpoR phosphorylation in primary erythroid cells

Since Lnk abrogated all 3 major signaling pathways originating from the EpoR, we analyzed whether Lnk also affects the phosphorylation of the EpoR and its associated JAK2 kinase. In control cells, Epo induced EpoR (Figure 7A) and JAK2 (Figure 7B) phosphorylation in 7 to 10 minutes; however, in wild-type Lnk-, but not Lnk (R364E)–transduced erythroid cells, both EpoR and JAK2 phosphorylation were markedly reduced (Figure 7A-B). In fact, Lnk itself becomes tyrosine-phosphorylated shortly following Epo stimulation, and this phosphorylation was abolished when the Lnk SH2 domain was disrupted (Figure 7C).

Therefore, the Lnk SH2 domain is required for its own phosphorylation, and is essential for attenuating EpoR and JAK phosphorylation induced by Epo.

Discussion

Lnk is a physiologic negative regulator of Epo-mediated signaling and erythropoiesis

Among mice nullizygous for Lnk family members, Lnk-deficient mice show the most profound perturbation in hematopoiesis. In addition to an abnormal expansion of immature B cells and enhanced BM repopulating activity, Lnk-deficient animals exhibit elevated numbers of megakaryocytes with increased ploidy.

In this study, we demonstrated that Lnk-deficient mice have increased numbers of erythroid progenitors in the BM and spleen. In addition, CFU-e progenitors from Lnk-deficient spleens are hypersensitive to Epo compared with wild-type controls. Lnk-deficient mice are less severely affected by and recover faster from erythropoietic stress, and this is due to an enhanced erythropoietic rate in Lnk-deficient mice after PHZ treatment. A principal if not the sole action of Lnk in erythropoiesis is the direct inhibition of Epo/EpoR signaling, since CD71+ erythroid progenitors from PHZ-treated Lnk-deficient spleens exhibited increased activation of Epo-induced signaling pathways. Thus, one of our most important results is that the adaptor protein Lnk is a physiologic negative regulator for Epo-mediated erythropoiesis.

Although Lnk−/− mice show superior recovery from erythropoietic stress and have increased erythroid progenitor numbers, they exhibit normal steady-state hematocrits. One possibility is that Lnk−/− mice have decreased Epo levels, which is technically difficult to quantify at normal or subnormal serum levels. The fact that Lnk deficiency does not result in enhanced EpoR signaling at the steady state (data not shown) may partially explain the lack of elevated red blood cell counts in these mice. Nonetheless, how the differentiation of BFU-e to CFU-e progenitors and subsequently to mature red blood cells is compensated in Lnk−/− mice remains to be elucidated. We have similar findings to Dr Tony Pawson’s group that in Lnk−/− mice Ter119+ cells were decreased in the BM but increased in the spleen. The balance or dynamics between BM and extramedullary hematopoiesis in Lnk−/− mice is unclear. Whether the BM environment is perturbed in Lnk-deficient mice, or abnormal lymphoid or myeloid homeostasis in these mice have an effect on BM erythropoiesis, remains to be determined.
Lnk inhibits Epo-dependent erythroblast differentiation

Lnk overexpression in primary erythroid progenitors blocks Epo/EpoR-dependent erythropoiesis, and the first manifestation (16 hours) is the abrogation of Epo-dependent hyper-up-regulation of the transferrin receptor CD71/TIR (R1 to R2). This occurs before Lnk-induced apoptosis, and in the absence of cell cycle arrest. By the end of a 2-day culture in the presence of Epo, Lnk overexpression resulted in fewer terminal differentiated erythrocytes, which appear to have less hemoglobin than control cultures. The fact that Lnk-expressing erythroblasts display an almost identical “differentiation” profile and cyto-logic morphology compared with control cells cultured in the absence of Epo, suggests that Lnk specifically inhibits Epo/EpoR signaling.

CD71/TIR, which acts in the cellular uptake of the iron-transferrin complex, is up-regulated in many cell types during proliferation. Due to an exceptionally large demand for iron to support hemoglobin production, erythroid cells express 100-fold higher levels of CD71/TIR than nonerythroid cells. The expression levels of CD71/TIR in erythroid cells couple with cell differentiation as well as proliferation. This hyper-up-regulation of CD71/TIR in early precursors (R1 to R2) is completely Epo-dependent, and mediated via TIR gene transcriptional and posttranscriptional mechanisms. In agreement with Epo-dependent CD71/TIR up-regulation, overexpression of constitutive active Stat5 in primary erythroblasts, one of the major signaling molecules induced by Epo, led to Epo-independent CD71/TIR up-regulation (data not shown).

Lnk induces erythroblast apoptosis in the absence of cell cycle arrest

Epo is believed to serve as a survival factor for committed CFU-e progenitors and allows them to undergo proliferation and terminal differentiation. Largely, apoptosis in erythroid progenitors deprived of Epo occurs during the G1 and S phases of the cell cycle, without growth arrest. Consistent with this, mice deficient for the antiapoptotic protein Bcl-Xl, which is a Stat5 target and induced by Epo in late erythropoiesis, lack mature erythrocytes. Conversely, exogenous expression of Bcl-Xl in primary erythroblasts allows terminal differentiation without Epo. Our findings in primary fetal liver cultures also agree with this antiapoptotic role of Epo. Lnk overexpression, which blocks Epo signaling, leads to a dramatic cell death but not cell-cycle arrest.

Erythroid progenitor cells possess a vast heterogeneity in their sensitivity to Epo. This intrinsic difference of erythroblasts in their sensitivity to Epo might be tightly regulated through negative signaling molecules associated with the EpoR. One example is the suppressor of cytokine signaling (SOCS) proteins. SOCS1-deficient fetal liver CFU-e progenitors exhibit an enhanced sensitivity to Epo in forming erythroid colonies. Similarly, we found that Lnk-deficient spleens are also hypersensitive to Epo in generating CFU-e colonies. In primary cultures of fetal liver cells, overexpression of SOCS1 in erythroid progenitors caused a dramatic cell death (data not shown), which is very similar to what we observed after Lnk overexpression. Taken together, our data suggest that Lnk is a negative regulator of Epo/EpoR signaling that controls the threshold of Epo-dependent survival.

Mechanism of Lnk inhibitory functions in cytokine receptor signaling pathways

C-Kit was previously reported to be an Lnk target. We recently identified a second Lnk target, the Tpo receptor mpl. We showed that Lnk-deficient megakaryocytes exhibited enhanced and prolonged activation of Tpo mpl-induced signaling proteins. In this study, we discovered a third Lnk target, the EpoR. We demonstrated that Lnk abrogates Epo-induced Stat5, Akt, and MAPK activation in both hematopoietic cell lines and primary erythroblasts. Consistently, PHZ-treated Lnk-deficient splenic erythroid progenitor cells showed enhanced signaling transduction induced by Epo.

One common feature identified in Lnk regulation of these 3 receptors is that the Lnk SH2 domain is crucial for its inhibitory function. Therefore, Lnk may negatively regulate signaling pathways originating from multiple cytokine receptors through a similar mechanism. Interestingly, the conserved tyrosine near the C-terminus of Lnk, Y536, is dispensable for the negative regulatory roles of Lnk in lymphocyte development and Tpo-mediated megakaryocytogenesis. In contrast, we found that Y536 is important, but not required, for modulating Epo-dependent 3D cell proliferation or erythroblast survival. The Lnk PH domain contributes but is not essential for inhibiting both Epo- and Tpo-mediated hematopoiesis, whereas it is dispensable for inhibiting SCF-dependent hematopoietic differentiation in AGM culture.

Taking advantage of the accessibility and abundance of fetal liver erythroid cells, we further demonstrated that Lnk itself becomes tyrosine phosphorylated following Epo administration and inhibits Epo-induced EpoR phosphorylation and JAK2 activation. Interestingly, Lnk does not inhibit EpoR cell-surface expression (Lily Huang and W.T., unpublished data, October 2003); neither does it induce EpoR or JAK2 degradation (Figure 7). Our data support several possible mechanisms by which Lnk downregulates JAK2 activity. One is that Lnk may disrupt the binding of positive regulators of JAK2, such as SH2-B. Second, Lnk may recruit other JAK2 inhibitors, such as SOCS1 or PTP-1B. Third, binding of Lnk to JAK2 may cause a conformational change that keeps JAK2 in a kinase-inactive state. Furthermore, the Lnk SH2 domain is essential for inhibiting EpoR and JAK2 phosphorylation, and the Lnk SH2 mutant lost its ability to be phosphorylated in response to Epo. These findings suggest that one or more tyrosine-phosphorylated proteins interact with the Lnk SH2 domain, and are crucial for phosphorylating Lnk and conferring Lnk inhibitory functions.

Taken together, we demonstrate that Lnk through its SH2 domain negatively regulates Epo-mediated erythropoiesis and Epo/EpoR signaling pathways by down-modulating JAK2 activation. Our findings likely provide a generalized mechanism for Lnk inhibitory function in the signaling of many type 1 cytokine receptors. This represents a new mechanism for rapidly down-regulating cytokine receptor signaling. Our work adds Lnk to the existing list of negative regulators of cytokine signaling including the SOCS proteins and SHP1 phosphatase.

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Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways

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