

Btk Is Required for an Efficient Response to Erythropoietin and for SCF-controlled Protection against TRAIL in Erythroid Progenitors

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Abstract

Regulation of survival, expansion, and differentiation of erythroid progenitors requires the well-controlled activity of signaling pathways induced by erythropoietin (Epo) and stem cell factor (SCF). In addition to qualitative regulation of signaling pathways, quantitative control may be essential to control appropriate cell numbers in peripheral blood. We demonstrate that Bruton's tyrosine kinase (Btk) is able to associate with the Epo receptor (EpoR) and Jak2, and is a substrate of Jak2. Deficiency of Btk results in reduced and delayed phosphorylation of the EpoR, Jak2, and downstream signaling molecules such as Stat5 and PLC γ 1 as well as in decreased responsiveness to Epo. As a result, expansion of erythroid progenitors lacking Btk is impaired at limiting concentrations of Epo and SCF. In addition, we show that SCF induces Btk to interact with TNF-related apoptosis-inducing ligand (TRAIL)-receptor 1 and that lack of Btk results in increased sensitivity to TRAIL-induced apoptosis. Together, our results indicate that Btk is a novel, quantitative regulator of Epo/SCF-dependent expansion and survival in erythropoiesis.

Key words: Jak2 • Stat5 • hematopoiesis • signal transduction

Introduction

Signals emanating from the receptors for erythropoietin (Epo) receptor and stem cell factor (SCF) (c-Kit) are crucial to positively regulate erythropoiesis. Epo or EpoR knockout mice die at day 12.5 of embryonic development with a lack of erythrocytes (1), whereas mice carrying mutations in c-Kit or SCF are severely anemic (2). In vivo, physiological Epo concentrations are suboptimal, causing apoptosis of ~20% of the erythroid cells present in bone marrow (3). This

apoptotic process may be further enhanced upon activation of "death receptors" (4) of which Fas and receptors for TNF α and TNF-related apoptosis-inducing ligand (TRAIL) are expressed in erythroid progenitors (5–7). The observed apoptosis is probably caused by interplay of apoptotic and survival factors. For instance, in myelodysplastic syndrome (MDS), the lack of erythrocytes has been attributed to a failure to respond to Epo as well as by enhanced sensitivity to death ligands (8, 9).

Ex vivo, erythroid progenitors can be expanded in the presence of Epo, SCF, and glucocorticoids, whereas Epo alone induces differentiation to erythrocytes (10–12). Activation of the EpoR and c-Kit results in phosphorylation of the receptors and activation of multiple signaling pathways (13–15). Common signaling pathways involve Src-like

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The online version of this article includes supplemental material.

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Abbreviations used in this paper: Btk, Bruton's tyrosine kinase; Dex, dexamethasone; Epo, erythropoietin; MDS, myelodysplastic syndrome; PKC, protein kinase C; RE, reexpressing; SCF, stem cell factor; TCL, total cell lysate; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR1, TRAIL receptor 1.

kinases, PI3 kinase, PLC γ 1 and ERK1/2, whereas Stat5 phosphorylation is only induced by Epo (for review see references 16, 17). Although the molecular mechanisms of Epo/SCF synergy in progenitor expansion are unknown, it may involve the specific activation of cooperating downstream effector molecules, or changes in signaling strength and/or duration. For instance, PI3 kinase activation of PKB/Akt is significantly higher after SCF stimulation and persists longer than Epo-induced PKB/Akt phosphorylation (reference 10 and unpublished data). Inhibition of PI3 kinase abrogates expansion of erythroid progenitors and induces differentiation instead (10).

PI3 kinase activity generates phosphatidylinositol-3-phosphates (e.g., PIP₃), which target proteins with a pleckstrin homology domain to the plasma membrane. This includes members of the Tec family of cytoplasmic tyrosine kinases (i.e., Bmx/Etk, Bruton's tyrosine kinase [Btk], Itk, and Tec; reference 18). Tec kinases are primarily expressed in hematopoietic lineages (19). Btk represents the most extensively studied member of the family. Btk mutations or deficiency cause X-linked agammaglobulinemia, a block in B cell development, in humans and the milder X-linked immunodeficiency phenotype in mice (20–22). Btk controls B cell receptor signaling and sensitivity to apoptosis induction. In DT40 cells, Btk associates with Fas and inhibits Fas-induced apoptosis (23), whereas Btk is required in the same cell type for radiation-induced apoptosis (24). Activation of Tec family kinases is largely dependent on the synergistic action of PI3 kinase and a Src-like kinase activity (25), although alternative pathways have been suggested (26). The PH-domain of Tec kinases imposes membrane recruitment (27), which results in phosphorylation on a tyrosine residue in the kinase domain (e.g., Y551 in Btk). The subsequent conformational change releases intramolecular inhibitory interactions, allows autophosphorylation within the SH3 domain (e.g., Y223 in Btk), and maximum kinase activity (28–30). Tec, as well as Btk, was also shown to associate with the non-Src-like kinases Syk, Jak1, and Jak2 in COS cells (31–33). Tec and Jak2 were shown to cross-phosphorylate each other (31, 32), but the physiological significance of this interaction has not been investigated.

Expression of Btk and Tec in erythroid cells has been described previously (34, 35), but so far only the role of Tec in c-Kit signaling has been studied in more detail (36). The analysis of erythroid progenitors derived from *wt* and Btk-deficient (Btk⁻) mice showed that Btk is required for progenitor expansion at low, physiological growth factor concentrations. At the molecular level, Btk enhanced Jak2 activity and loss of Btk resulted in reduced Epo-induced phosphorylation of EpoR, Jak2, Stat5a/b, and PLC γ 1. In addition, Btk⁻ progenitors are highly sensitive to TRAIL-induced apoptosis because they lack SCF-induced association of Btk with TRAIL receptor 1 (TRAILR1).

Materials and Methods

Antibodies, Growth Factors, and Plasmids. Rabbit antisera against Jak2, a mouse monoclonal antibody recognizing phos-

pho-Stat5a/b (Tyr694-Stat5a and Tyr 699-Stat5b) and antibodies for TRAILR1/2 were obtained from Upstate Biotechnology. Rabbit antisera recognizing the mouse EpoR, PLC γ 1, Btk, c-Kit, and the antiphosphotyrosine mouse monoclonal antibody PY99 were obtained from Santa Cruz Biotechnology, Inc. Phospho-Btk (Tyr223) was obtained from Cell Signaling. Anti-Actin antibody was obtained from Sigma-Aldrich. Recombinant human Epo was a gift from Ortho-Biotech. Dexamethasone (Dex) was purchased from Sigma-Aldrich; mouse SCF and recombinant TRAIL was obtained from R&D Systems. The complete open reading frame of Btk and Btk kinase dead (K430R, a gift from J. Borst, Netherlands Cancer Institute, Amsterdam, Netherlands), Tec, Jak2, Lyn, c-Kit, and EpoR was cloned into the mammalian expression vector pSG5 (Stratagene). Additionally, Btk was cloned into the retroviral vector pBabe-puro.

Cell Lines, Primary Erythroid Cultures, Transfections, and Viral Transductions. COS and ecotropic Phoenix (ϕ E) cells were maintained in DMEM supplemented with 10% FCS (Life Technologies). For COS transfection experiments, 10⁶ cells were seeded in 60-mm dishes, transfected with 12 μ g DNA for 3–4 h, washed, and harvested after 48 h as described previously (36). Primary fetal liver and bone marrow erythroid progenitors as well as erythroid bone marrow cell lines (see Online Supplemental Material section; reference 37) were cultured in StemPro-34TM medium (Life Technologies) supplemented with 2 U/ml Epo, 100 ng/ml SCF, and 1 μ M Dex, unless indicated differently. Cell cultures were maintained at 1.5–3 \times 10⁶ cells/ml. To induce terminal erythroid differentiation, cells were washed in PBS and reseeded at 2–3 \times 10⁶ cells/ml in StemPro-34TM medium containing insulin (4 \times 10⁻⁴ IE/ml; Actrapid HM), the Dex antagonist ZK-112993 (3 \times 10⁻⁶ M; reference 12), 1 mg/ml of iron-saturated human transferrin (Sigma-Aldrich), and various concentrations of Epo as indicated.

Viral transduction of the Btk⁻ erythroid bone marrow cell line 3G4 was performed as follows: ϕ E cells were transfected as described for COS cells. After 48 h of transfection, cells were treated with 10 μ g/ml mitomycin C (Kyowa) for 1 h, washed 3 times with PBS, left untreated for 4 h, and washed; 3G4 cells were added (0.5 \times 10⁶ cells/transfection) to be cocultured in StemPro-34TM medium supplemented with growth factors as aforementioned. After 48 h, the erythroid cells were transferred from the ϕ E cell layer to a new dish and selected for stable transfectants with 2 μ g/ml puromycin (Sigma-Aldrich). To obtain single cell-derived clones, cells were seeded in semi-solid StemPro-34TM medium supplemented with Epo, SCF, and Dex plus puromycin (2 μ g/ml).

Immunoprecipitations and Western Blotting. Primary erythroid cells and the erythroid bone marrow cell lines were factor deprived in plain IMDM (Life Technologies) for 4 h at 5 \times 10⁶ and restimulated for 10 min at 37°C (40–80 \times 10⁶/ml) with 500 ng/ml SCF, 5 U/ml Epo, 200 ng/ml TRAIL, or combinations of factors at concentrations indicated in the figure legends. To stop the reaction, 10 volumes of ice-cold PBS were added. Cell lysis, immunoprecipitation, SDS-PAGE, and Western blotting were performed as described previously (36). Membranes were stripped in 63 mM Tris-HCl, pH 6.1, 2% SDS, and 100 mM β -mercaptoethanol for 30 min at 50°C after which they were reblocked and restained.

Hemoglobin Content Determination and Cell Morphology. Small aliquots of the cultures were removed and analyzed for hemoglobin content by photometry as described previously (38). The values described were the average of triplicate measurements after normalization for cell number and mean single cell volume. For

histological analysis, erythroid cells at various stages of cell culture were cytocentrifuged onto glass slides and subsequently stained with histological dyes and neutral benzidine for hemoglobin as described previously (39). Nucleated and enucleated erythrocytes (brown or yellow stained, small cells), partially mature or immature cells (larger cells with gray or blue cytoplasm), and dead cells (fragmented/condensed nuclei, disintegrated cells) were counted under a microscope, evaluating >600 cells on multiple, randomly selected fields per sample (49, 51). Images were taken using a CCD camera and were processed with Adobe Photoshop.

Membrane Expression of Various Receptors. Flow cytometric analysis was performed essentially as described previously (22). Antibodies recognizing c-Kit, TER119, and Integrin α 4 were obtained from BD Biosciences. Biotinylated Epo was made using the biotin labeling kit (cat. no. 1418165; Roche) following the manufacturer's protocol.

Online Supplemental Material. Online supplemental material describes (a) how the bone marrow-derived cell lines used in these studies were established, (b) peripheral blood parameters and colony-forming progenitors present in the fetal liver of *wt* and Btk-deficient mice (Table S1), and (c) the response of *wt* and Btk-deficient mice to hypoxia (phenylhydrazine-induced stress erythropoiesis in the spleen; Fig. S1). Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20031109/DC1>.

Results

Btk Is Phosphorylated in Response to Epo and SCF. To address whether Btk has a functional role in Epo/SCF-dependent erythropoiesis, we tested if Btk is tyrosine phosphorylated in response to Epo or SCF. Erythroid progenitors expanded from E12.5 mouse fetal liver were factor deprived and restimulated with Epo and SCF. Epo as well as SCF induced tyrosine phosphorylation of the Btk autophosphorylation site Y223 with similar kinetics and intensity, whereas costimulation with both Epo and SCF resulted in a similar phosphorylation intensity of Btk (Fig. 1 A). Phosphorylation of Btk was also detected with the antiphosphotyrosine antibody PY99 (unpublished data). Because activation of Btk in B cells is dependent on PI3 kinase and Src-like kinases, we examined the presence of a similar mechanism in erythroid cells. Immortalized erythroid progenitors were factor deprived and restimulated in the presence of increasing inhibitor concentrations suppressing PI3 kinase (LY294002 [LY]) or Src-like kinases (PP2). Btk tyrosine phosphorylation was dose-dependently inhibited by LY with complete inhibition at 30–45 μ M (Fig. 1 B). Inhibition of Src family kinases with PP2 (0.1–5 μ M) did not result in an inhibition of Btk tyrosine phosphorylation (Fig. 1 C, top rectangle, bottom band). Inhibition of (unknown) tyrosine-phosphorylated proteins in total cell lysates by PP2 indicated that the inhibitor was functional. Unfortunately, the Src kinase inhibitors cannot be used to examine whether SCF-induced Btk phosphorylation is dependent on Src kinases because they directly inhibit c-Kit kinase activity (reference 40 and unpublished data). In conclusion, Epo induces PI3 kinase-dependent and Src kinase-independent activation of Btk in erythroid progenitors.

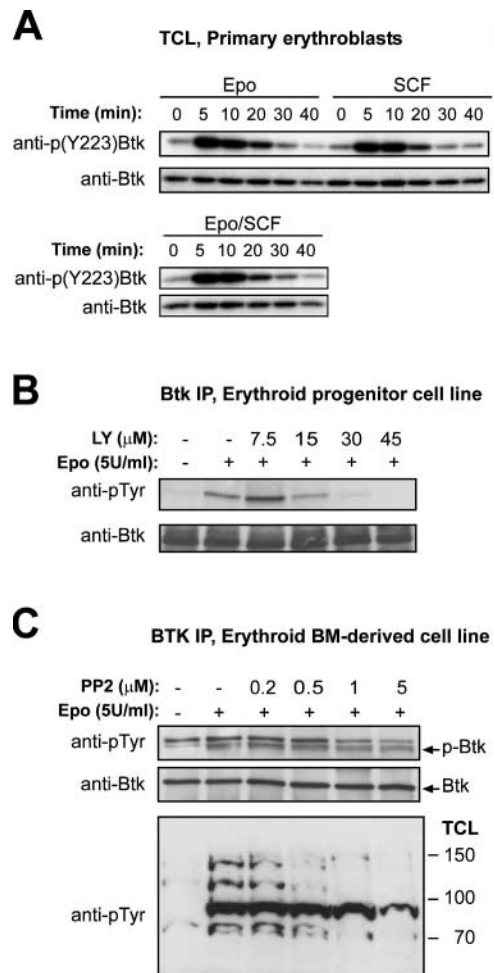


Figure 1. Epo and SCF induce Btk tyrosine phosphorylation. (A) Primary, fetal liver-derived progenitors were factor deprived and restimulated with Epo, SCF, or Epo/SCF as shown. Stimulation time is indicated in minutes. Btk phosphorylation was analyzed using an anti-Y223 phospho-specific antibody. Total Btk was stained to confirm equal loading. (B and C) *wt* erythroid progenitor cells (2B6) were factor deprived in the absence or presence of various concentrations of the PI3 kinase inhibitor LY294002 (B, LY) or the Src kinase inhibitor PP2 (C) as shown and restimulated with Epo for 10 min. Btk immunoprecipitates were analyzed for phosphorylation using antiphosphotyrosine antibodies (PY99; p-Btk is the bottom band) and restained for total Btk (B) or for phosphorylated proteins in total cell lysates (TCLs) as a control (C). Size markers are indicated in kilodaltons.

Btk Interacts with the EpoR and Jak2 and Is Tyrosine Phosphorylated by Jak2. The Epo-dependent activation of Btk suggests that Btk may interact with the EpoR and/or Jak2. Btk was expressed in COS cells together with Jak2 and/or the EpoR. Jak2 efficiently coimmunoprecipitated with Btk (Fig. 2 A). Btk also coimmunoprecipitated with the EpoR, but was not able to phosphorylate the EpoR in the absence of Jak2 (Fig. 2 B). Because Epo-induced Btk phosphorylation is independent of Src kinases, we tested whether Jak2 or other kinases associated with the EpoR complex are able to phosphorylate Btk. A kinase-dead mutant of Btk (K430R) was expressed in COS cells together with Jak2, Lyn, or c-Kit. Jak2 and Lyn coimmunoprecipitated with Btk and phosphorylated Btk on tyrosine

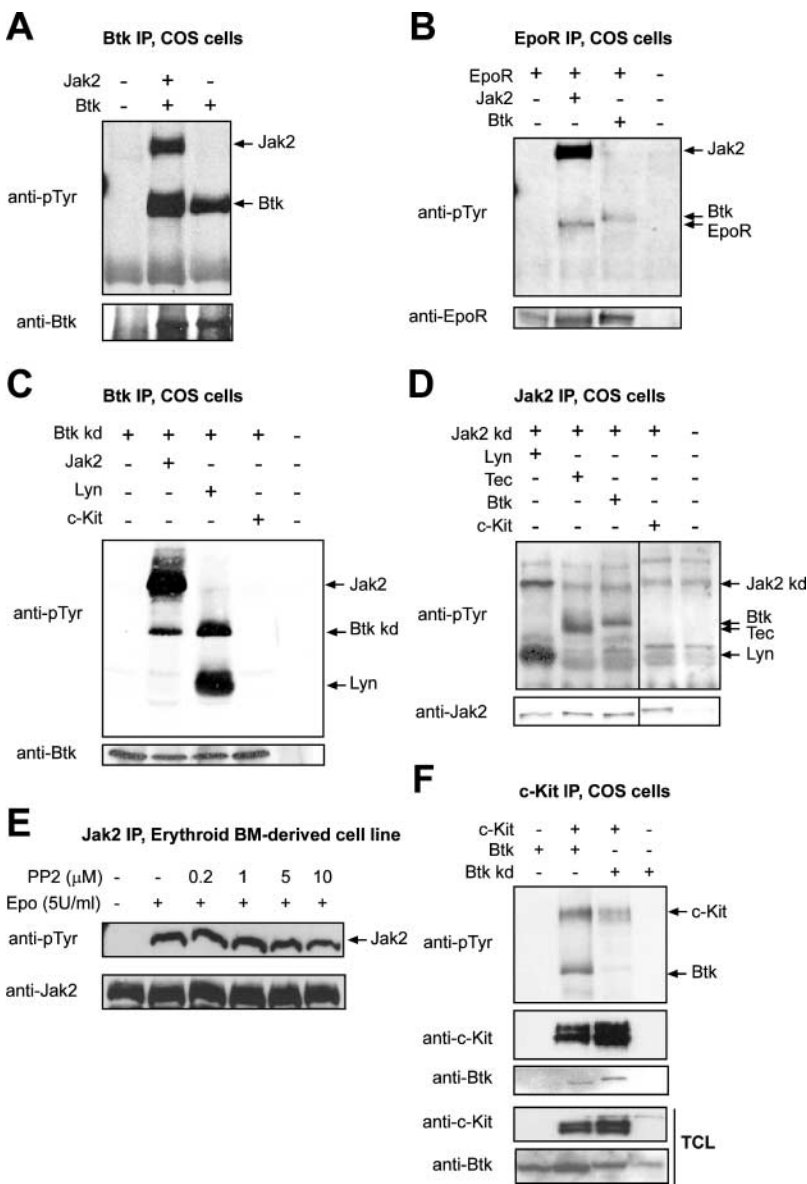


Figure 2. Btk coimmunoprecipitates with and is a substrate of Jak2. (A–D and F) COS cells were transfected with pSG5-based expression constructs encoding the indicated proteins. Expression of all proteins was verified in Western blots using specific antibodies (not depicted). After 48 h, cells were stimulated with 5 U/ml Epo (B) or 500 ng/ml SCF (C, lane 4; D and E, lane 4) for 10 min, harvested, and lysed, and Btk (A and C), EpoR (B), Jak2 (D), and c-Kit (F) were immunoprecipitated. (top panels) Immunoblots were stained with antiphosphotyrosine antibodies (PY99). The blots were restained with antibodies recognizing the immunoprecipitated and coimmunoprecipitating proteins to check for equal loading. Arrows indicate the position of the immunoprecipitated and coimmunoprecipitating proteins. (E) *wt* erythroid progenitors (2B6) were factor deprived in the absence or presence of the Src kinase family inhibitor PP2 as indicated and stimulated with Epo for 10 min. Epo-induced Jak2 phosphorylation was assayed on immunoblots using PY99 (top). (bottom) Blots were restained with anti-Jak2 to confirm equal loading. (F) c-Kit immunoprecipitates were stained with antiphosphotyrosine antibodies (PY99), and blots were restained with anti-c-Kit and anti-Btk antibodies, whereas total cell lysates (TCLs) were stained with anti-c-Kit and anti-Btk to check expression levels.

(Fig. 2 C). To analyze whether, in reverse, Btk can phosphorylate Jak2, a kinase-dead mutant of Jak2 (K1114M) was expressed in COS cells together with Lyn, Btk, Tec, or c-Kit. Although Lyn efficiently phosphorylated Jak2, Jak2 was not phosphorylated by Btk (Fig. 2 D). In contrast to papers by others (31, 32), Tec was also not able to phosphorylate Jak2 in COS cells. In conclusion, Btk can coimmunoprecipitate with the EpoR and Jak2, and Btk can serve as a substrate for Jak2. This may explain the PP2-resistant, Epo-induced Btk phosphorylation because PP2 did not affect Epo-induced Jak2 tyrosine phosphorylation (Fig. 2 E). To test whether c-Kit directly associates with and phosphorylates Btk, we coexpressed c-Kit with either Btk or Btk (K430R). Although both *wt* and kinase-dead Btk (K430R) coimmunoprecipitated with c-Kit, only *wt* Btk was efficiently stained by antiphosphotyrosine antibody (Fig. 2 F). This suggests that c-Kit cannot directly phosphorylate

Btk and that SCF-induced phosphorylation of Btk uses additional kinases.

Btk⁻ Erythroid Progenitors Are Defective in Proliferation, But Not in Differentiation. Because we found Btk to be a substrate of Epo/SCF signaling, we compared factor-dependent survival, expansion, and differentiation of *wt* and Btk⁻ progenitors. Fetal liver cells from E12.5 Btk⁻ and *wt* littermates were cultured in medium supplemented with Epo, SCF, and Dex. In both cultures, progenitors proliferated with similar kinetics (Fig. 3 A). Because standard growth factor concentrations (2 U/ml Epo and 100 ng/ml SCF) exceed physiological concentrations to ensure long-term expansion of progenitors at maximal proliferation rate, we also examined expansion of *wt* and Btk⁻ cells at lower concentrations of Epo (0.5 U/ml) and SCF (25 ng/ml). Although *wt* progenitors could be expanded for at least 10 d at reduced growth factor concentrations, cultures of Btk⁻

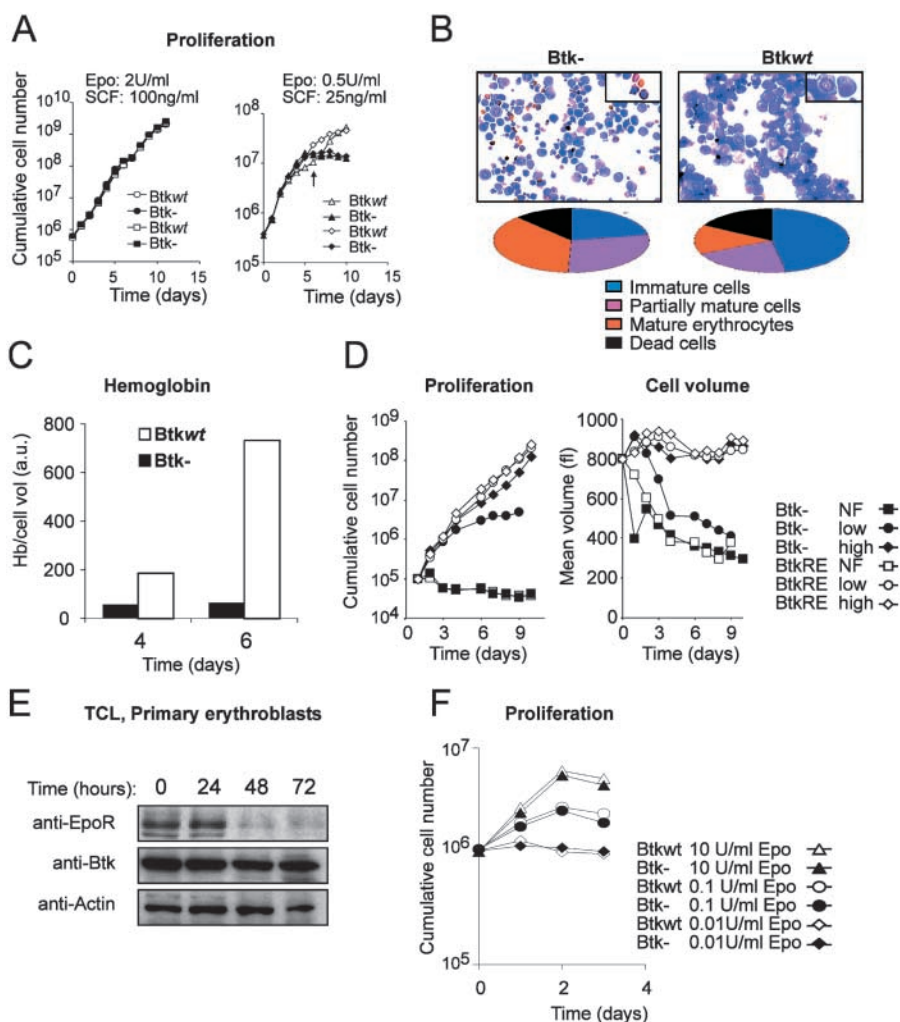


Figure 3. Btk^{-} erythroid progenitors show enhanced differentiation at the expense of renewal. (A) Btk^{-} primary fetal liver progenitors were seeded in medium containing high (2 U/ml Epo and 100 ng/ml SCF) or low (0.5 U/ml Epo and 25 ng/ml SCF) concentrations of SCF and Epo, while maintaining normal concentrations of Dex, and cumulative cell numbers were determined. The different symbols represent two independent experiments using *wt* and Btk^{-} erythroid progenitors as indicated. (B) Histological analysis of *wt* and Btk^{-} cells described in A at day 6 (A, arrow). The percentages of erythroid cells at different stages of maturity (as indicated) were scored for *wt* and Btk^{-} cultures and plotted in a pie chart. Percentage of immature cells: *wt*, 47%, and Btk^{-} , 22%. Partially mature cells: *wt*, 21%, and Btk^{-} , 28%. Mature cells: *wt*, 15%, and Btk^{-} , 37%. Dead cells: *wt*, 17%, and Btk^{-} , 12%. (C) Hemoglobin content (Hb/cell volume in arbitrary units) of cells as described in A was measured at days 4 and 6. (D) Btk^{-} bone marrow cell lines ectopically Btk -reexpressing ($BtkRE$) and Btk^{-} cell lines (Btk^{-}) were treated as described in A. Open symbols represent $BtkRE$, and closed symbols represent Btk^{-} cells cultured on low, high, or no-growth factors (NF; see A). (E) Primary fetal liver progenitors were seeded in differentiation medium. Expression of Btk and the $EpoR$ during differentiation was checked by Western blotting using Btk and $EpoR$ antibodies. Equal loading was examined using an Actin antibody. (F) Cells were seeded in differentiation conditions at 10, 0.1, and 0.01 Epo U/ml. Cumulative cell numbers were determined. Symbols indicate the different growth conditions as well as *wt* and Btk^{-} cultures.

progenitors became stationary at day 6 (Fig. 3 A). Analysis of cell morphology and hemoglobin staining of Btk^{-} cultures at day 6 showed an increase in mature erythrocytes at the expense of erythroid progenitors as compared with *wt* cells (Fig. 3 B). The same was evident by the much smaller average cell size exhibited by Btk^{-} cells compared with *wt* cells, determined using an electronic cell counter (unpublished data). Concordantly, Btk^{-} cultures showed an increase in hemoglobin levels compared with *wt* cultures (Fig. 3 C, 3-fold at day 4; 11-fold at day 6). In conclusion, Btk deficiency abrogates the expansion capacity of erythroid progenitors exposed to limiting concentrations of Epo and SCF, favoring terminal differentiation at the expense of erythroid progenitor expansion.

To study Btk^{-} cells in detail, including biochemical assays, we established immortal cultures of p53-deficient Btk^{-} and $Btkwt$ bone marrow-derived erythroid progenitors (see Supplemental Material). At standard concentrations of Epo and SCF, $Btkwt$ (2C6) and Btk^{-} (3E8) immortal erythroid cultures could be similarly expanded and retained a cell volume of ~ 800 fl. In contrast, decreased concentrations of Epo (0.5 U/ml) and SCF (25 ng/ml) caused rapid cell size reduction and growth arrest in the

Btk^{-} progenitors. Re-expression of Btk in Btk^{-} cells restored the expansion capacity and blastlike cell size at reduced growth factor concentrations (Fig. 3 D).

The observation that progenitor expansion was impaired in Btk^{-} cells, whereas progenitors underwent terminal differentiation instead, suggests that Btk is not essential for differentiation. However, Btk expression is maintained during terminal differentiation, whereas $EpoR$ expression decreases during the later stages of differentiation (Fig. 3 E). To investigate whether Btk is required during Epo-induced terminal erythroid differentiation, fetal liver-derived primary *wt* and Btk^{-} progenitors were seeded in differentiation medium containing varying concentrations of Epo (0.01–10 U/ml). Although proliferation during differentiation showed an expected dependence on the Epo concentration, no differences between differentiating *wt* and Btk^{-} cultures were observed with respect to proliferation kinetics, hemoglobin content, or cell morphology (Fig. 3 F and not depicted).

EpoR Signaling Is Perturbed in Btk^{-} Erythroid Progenitors. To unravel how Btk^{-} deficiency could impair Epo/SCF-dependent progenitor expansion, we analyzed $EpoR$ and *c-Kit* signaling. $Btkwt$ (2C6) and Btk^{-} (3E8) immortalized progenitors were factor deprived and restimulated with 5

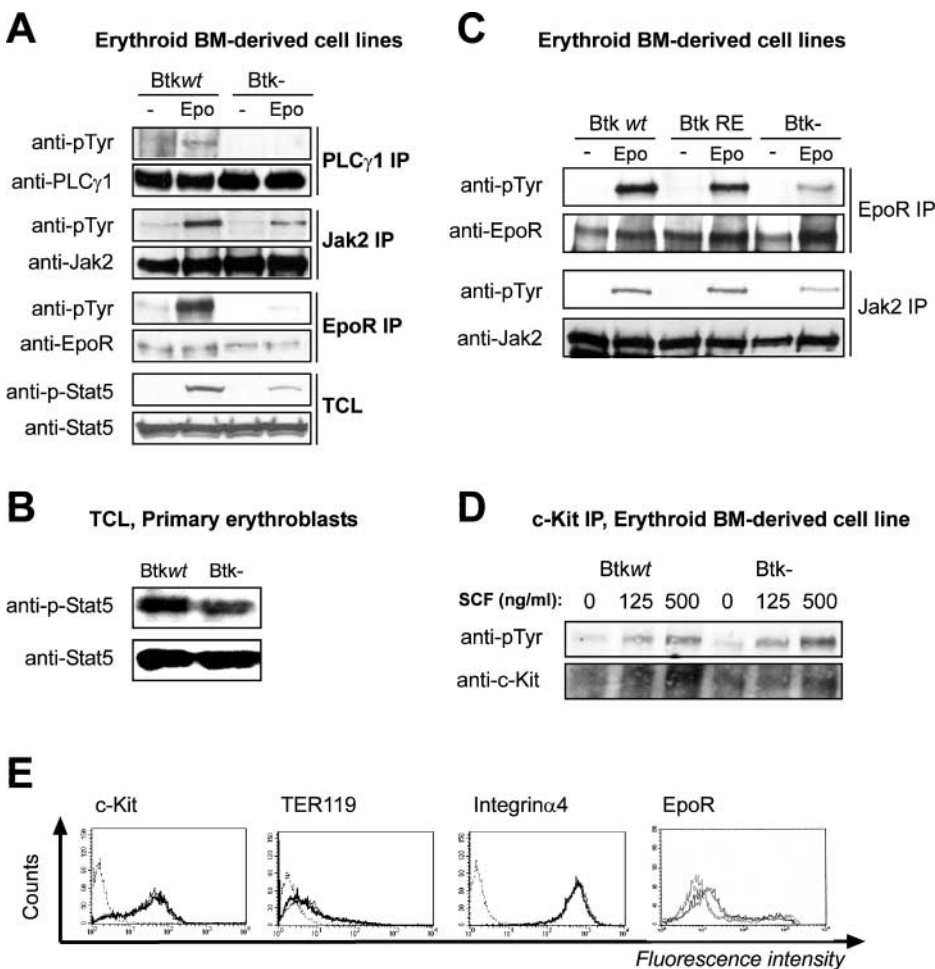


Figure 4. *Btk*⁻ cells are perturbed in Epo-induced signaling. (A–D) *wt* (*Btkwt*), *Btk*⁻ (*Btk*⁻), *Btk*-reexpressing (*BtkRE*) bone marrow–derived erythroid cell lines (A, C, and D), and primary *wt* and *Btk5*⁻ fetal liver progenitors (B) were factor deprived and restimulated with 5 U/ml Epo or SCF as indicated for 10 min. Cells were lysed, and the immunoprecipitated proteins as well as total cell lysates (TCLs) were analyzed by Western blotting. (top) Blots were stained with the antiphosphotyrosine antibody PY99, except for the TCLs (B), which were stained in B with anti-phospho-Tyr694/699 Stat5a/b. (bottom) The same blots were restained with antibodies recognizing the immunoprecipitated protein as indicated or with anti-total Stat5a/b for the TCLs. (E) Flow cytometric analysis of cell surface expression of c-Kit, TER119, Integrin α 4 by specific antibodies, and EpoR by biotinylated Epo in *wt* (thick black line) and *Btk*⁻ (thin black line) erythroid progenitors. Expression levels are also shown as histograms depicting control stainings (dotted line) and addition of excess Epo (gray line) for EpoR detection. Mean fluorescence values varied <10% for each staining in three independent experiments, indicating that *wt* and *Btk*⁻ erythroid progenitors express similar levels of the respective cell surface markers.

U/ml Epo. Epo-induced phosphorylation of PLC γ 1 was completely abrogated in *Btk*⁻ cells. Surprisingly, Epo-induced phosphorylation of the EpoR, Jak2, and Stat5, was also significantly reduced in the *Btk*⁻ cells compared with *wt* cells (Fig. 4 A). Epo-induced Stat5 phosphorylation was similarly reduced in primary progenitors (Fig. 4 B and not depicted). Re-expression of *Btk* in *Btk*⁻ progenitors restored the phosphorylation levels of the EpoR, Jak2, and Stat5 to those in *wt* cells (Fig. 4 C). Although *Btk* was also phosphorylated in response to SCF, SCF-induced phosphorylation of c-Kit was not reduced in absence of *Btk* (Fig. 4 D). To rule out that the impaired Epo-induced signal transduction in *Btk*⁻ cells was due to decreased cell surface expression of the EpoR, expression of the EpoR and c-Kit was determined by flow cytometry of nonpermeabilized cells. Surface expression of the EpoR and c-Kit was identical between *wt* and *Btk*⁻ erythroid progenitors, using the erythroid markers TER119 and Integrin α 4 as controls (Fig. 4 E).

***Btk* Controls Signaling Kinetics.** To examine whether the reduced response to Epo in *Btk*⁻ cells could be caused by altered signaling kinetics, the immortalized *Btkwt* (2C6), *Btk*⁻ (3E8), and *Btk*-reexpressing (RE) erythroid cells were factor deprived and restimulated with Epo for 5, 10, 60, and 120 min; and Stat5 phosphorylation was assessed. Consistently, Stat5 was rapidly phosphorylated in response to Epo

in *wt* and *BtkRE* cells, whereas phosphorylation in *Btk*⁻ cells was largely impaired. However, at late time points, similar levels of Stat5 phosphorylation were observed in all cell types (Fig. 5 A). Because Jak2 phosphorylates Stat5, Jak2 was immunoprecipitated from cell lysates generated at increasing time intervals after Epo stimulation of *wt* (2C6) and *Btk*⁻ (3E8) cells. Maximal phosphorylation of Jak2 was observed at 10–20 min after Epo stimulation in *wt* cells and only at 40 min in *Btk*⁻ cells (Fig. 5 B; the exposure of the blots was adapted to show [de]activation kinetics for *Btk*⁻ and *wt* cells, requiring much longer exposure of the PY blots in the *Btk*⁻ panel). Also, phosphorylation of the EpoR, though drastically reduced in *Btk*⁻ cells, is less rapidly down-regulated (Fig. 5 C; requiring again different PY exposures of *Btk*⁻ and *wt* panels). Analysis of Epo-induced Stat5 phosphorylation kinetics in primary bone marrow–derived erythroid progenitors confirmed the results obtained with the cell lines; Stat5 phosphorylation decreased after 20 min in *wt* cells, whereas it increased up to 90 min after Epo stimulation in *Btk*⁻ cells (Fig. 5 D).

***Btk* Regulates the Ligand Responsiveness of the EpoR.** Because *Btk* controlled the efficiency of EpoR signaling in time, it may also control the responsiveness to critical Epo concentrations. Immortalized *Btkwt* (2C6), *Btk*⁻ (3E8), and *BtkRE* cells were factor deprived and restimulated with Epo

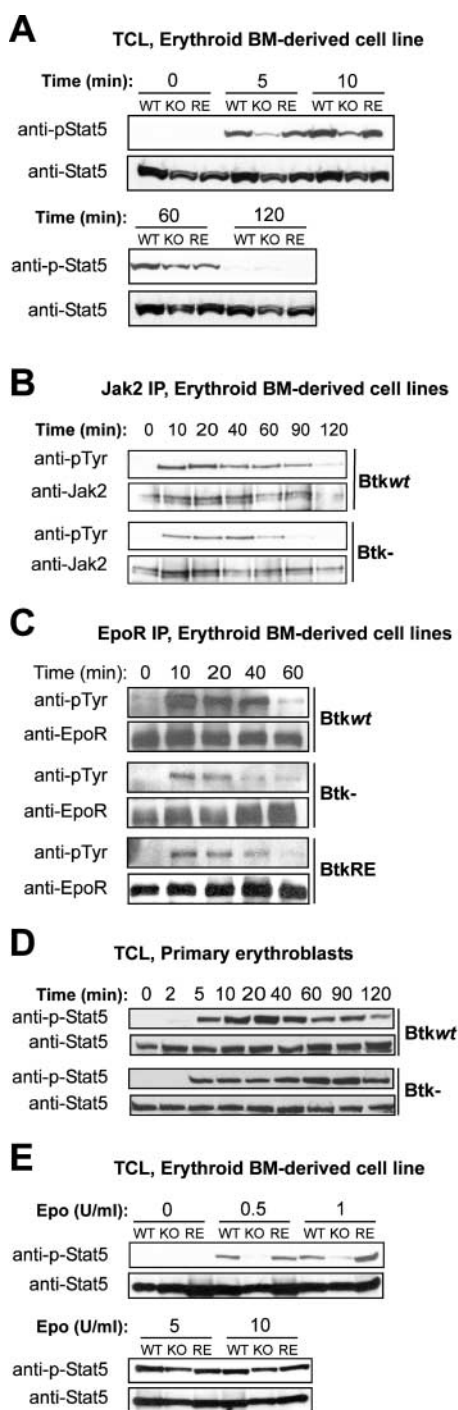


Figure 5. Btk⁻ erythroid progenitors have a delayed Epo-induced Jak2 and Stat5 phosphorylation. (A–E) *wt* (Btkwt), Btk⁻ (Btk⁻), and Btk-reexpressing (BtkRE) erythroid cell lines (A–C) or primary fetal liver progenitors (D) were factor deprived and restimulated with 5 U/ml Epo for the indicated times. Cells were lysed, and Jak2 (B) or the EpoR (C) was immunoprecipitated. The immunoprecipitations as well as total cell lysates (TCLs) were analyzed by Western blotting. (A and D) Blots were stained with anti-phospho-Tyr694/699 Stat5a/b (top) and restained with anti-total Stat5a/b to confirm equal loading (bottom). (B and C, top) Blots were stained with antiphosphotyrosine PY99. (bottom) The same blots were restained with anti-Jak2 or anti-EpoR to confirm equal loading. PY-staining of these proteins in Btk⁻ cells was much lower than in wt cells. Therefore, Btk⁻ panels were exposed much longer than wt panels

at concentrations ranging from 0.5 to 10 U/ml. Stat5 phosphorylation became detectable in *wt* and Btk reexpressing cells at 0.5 U/ml Epo, whereas similar levels were reached in Btk⁻ cells using 5 U/ml. With increasing levels of Epo, the decrease in Epo-induced Stat5a/b phosphorylation in Btk⁻ cells became markedly less apparent (Fig. 5 E). Together, the data show that Btk may have an important role in the regulation of signaling efficiency both in time as well as in response to suboptimal physiological concentrations of Epo.

Expansion of Btk⁻ Erythroid Progenitors Is Inhibited by TRAIL. Reduced sensitivity for Epo and hypersensitivity for “death ligands” is associated in several pathological conditions (9). Notably, loss of Btk has been shown to render B cells more sensitive to Fas-induced apoptosis in response to certain death signals (23, 41). To examine whether the reduced responsiveness to Epo in Btk⁻ cells affects death ligand-induced apoptosis in primary erythroid progenitors, we expanded primary *wt* and Btk⁻ erythroid progenitors from fetal liver in presence of various death-inducing ligands. INF γ killed both *wt* and Btk⁻ progenitors with identical kinetics, whereas the addition of 20 ng/ml FasL or 10 ng/ml TNF α under optimal and suboptimal proliferation conditions had no effect on *wt* or Btk⁻ cells (unpublished data). However, under optimal proliferation conditions, the addition of TRAIL (20 ng/ml) impaired the expansion of Btk⁻ progenitors, whereas *wt* control cells remained unaffected (Fig. 6 A). Morphological analysis indicated the presence of pycnotic cells (Fig. 6 B). On Western blots, Btkwt and Btk⁻ cells express similar levels of TRAILR1, indicating that increased receptor expression is not the cause of the enhanced TRAIL sensitivity (Fig. 6 C). Expression of TRAILR2 was not observed in erythroid progenitors (Fig. 6 C).

It has been reported previously that Btk can interact with Fas (23), whereas the Tec family member Bmx/Etk undergoes ligand-independent interaction with TNF α R2 (42). These interactions protected the cells from death ligand-induced apoptosis and allowed TNF α -induced cellular migration and tube formation in epithelial cells. Therefore, we tested whether Btk can interact with TRAILR1 and whether this interaction is dependent on either Epo or SCF. Progenitors expanded from *wt* fetal livers were factor deprived and subsequently restimulated with Epo, SCF, and/or TRAIL. Btk immunoprecipitates were analyzed for the presence of TRAILR1. Both SCF and TRAIL, but not Epo, induced an interaction of Btk and TRAILR1. In addition, costimulation of SCF and TRAIL resulted in a significantly enhanced TRAILR1 recruitment to Btk compared with SCF or TRAIL alone (Fig. 6 D). This suggests that SCF may inhibit TRAIL-induced apoptosis through enhancement of an interaction between the TRAILR1 and Btk, which may cooperate with reduced Epo responsiveness.

to enable proper comparison of kinetics of deactivation. (E) Erythroid progenitors were restimulated with increasing concentrations of Epo for 10 min as indicated. (top) Blots stained with anti-phospho-Tyr694/699 Stat5a/b; (bottom) the same blots were restained with anti-total Stat5a/b.

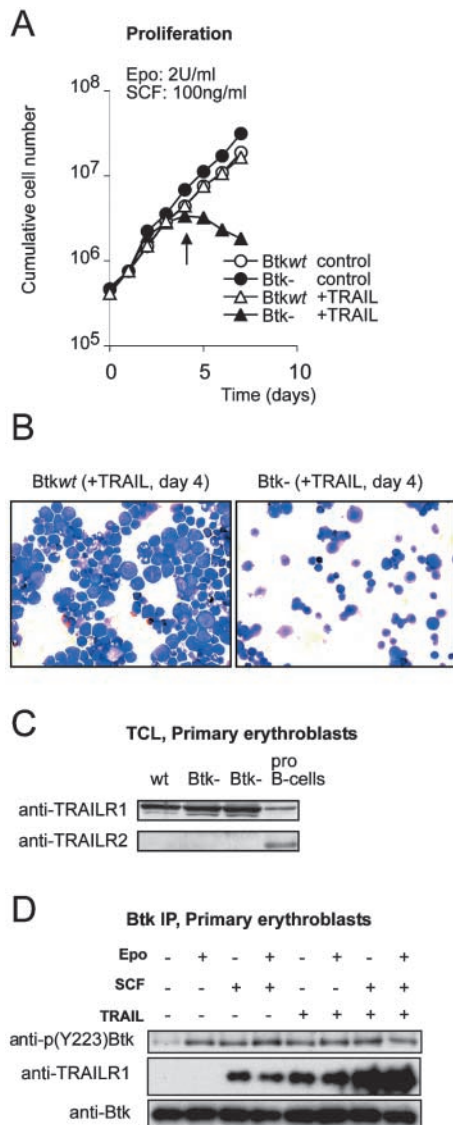


Figure 6. Btk^{-} erythroid progenitors are no longer protected by SCF from TRAIL-induced apoptosis. (A) *wt* and Btk^{-} fetal liver cells were seeded in medium containing Epo, SCF, and Dex in the presence and absence of 20 ng/ml TRAIL, and cumulative cell numbers were determined. (B) At day 4, cytopspins were prepared from cultures of *wt* and Btk^{-} cells in the presence of TRAIL (A, arrow) and stained with neutral benzidine plus histological dyes. (C) Western blots from cell lysates of *wt* and Btk^{-} primary fetal liver erythroid progenitors and primary pro-B cells were performed using anti-TRAILR1 and anti-TRAILR2 antibodies as indicated. (D) *wt* primary fetal liver progenitors were factor deprived and restimulated (for 10 min) with 5 U/ml Epo, 500 ng/ml SCF, and 200 ng/ml TRAIL or combinations of factors as indicated. Btk tyrosine phosphorylation was analyzed using an anti-phospho-Tyr223 Btk antibody (top), whereas coimmunoprecipitation of TRAILR1 was checked with anti-TRAILR1 (middle). Equal loading was confirmed by restaining the same blot with total anti-Btk (bottom).

Discussion

Of all circulating blood cells, the maintenance of circulating erythrocytes at constant, optimal numbers is most important. Physiological levels of the major erythroid sur-

vival and expansion factor Epo are suboptimal (<30 U/l in mice), and only part of the progenitors survives in the bone marrow to produce mature erythrocytes. In this paper, we show that the cytoplasmic tyrosine kinase Btk is required for an optimal response of erythroid progenitors to Epo, and for SCF-mediated survival in presence of TRAIL.

Btk Is a Target of Jak2 and Controls the Efficacy of EpoR Signaling. Stimulation of erythroid progenitors with Epo and SCF induced phosphorylation of Btk on its autophosphorylation site Y223, indicating that catalytic activity of Btk is enhanced after Epo and SCF stimulation. Interestingly, Jak2 and Lyn could phosphorylate Btk. Similarly, the Btk family member Tec is mainly phosphorylated by Lyn, but can also be phosphorylated by Jak1, Jak2, or Syk (31–33). Because the Src kinase inhibitors PP1 and PP2 directly inhibit c-Kit catalytic activity (reference 40 and unpublished data), we did not investigate whether SCF-mediated Btk activation requires Lyn. However, this is likely because c-Kit did not efficiently phosphorylate kinase-dead Btk (Fig. 2, E and F), whereas c-Kit is constitutively associated with Lyn in erythroid cells (36). Notably, Lyn phosphorylates Tec in response to SCF, and the same mechanism may hold for Btk.

Btk was expected to be required for the phosphorylation and activation of selected Epo-dependent signaling intermediates like PLC γ 1, a known downstream target of Tec kinases. However, the role of Btk in the control of Epo responsiveness was surprising. In the absence of Btk, Epo-dependent phosphorylation of Jak2 and the EpoR required increased levels of Epo or prolonged exposure to Epo. In contrast, activation of the SCF receptor was not perturbed. These data suggest that Btk may activate a signaling pathway that acts as an enhancer of Jak2 activity. Such pathways have not been described to date, but Btk does control several signaling pathways that could be candidates for such a function. First, Btk is required to activate PLC γ , whose main effectors are classical protein kinase Cs (PKCs) of which only PKC α is expressed in erythroid progenitors (43). We have shown previously that inhibition of PKC α does severely impair Epo-induced signal transduction similar to what we observe in Btk^{-} cells (43). To test whether the potential absence of a PLC γ -PKC α positive feedback loop in Btk^{-} cells could be responsible for their impaired EpoR signaling, we examined whether direct activation of PKC could restore a *wt*-like Epo responsiveness of EpoR signaling in these cells. Both bryostatin and phorbol ester enhanced Epo-induced phosphorylation of Jak2 and the EpoR in Btk^{-} cells, but the degree of enhancement was not significantly stronger than in *wt* cells and did not fully restore the Btk^{-} signaling defect. Second, because Btk associates with Jak2, it may directly affect the Jak2-associated protein complex. The Jak2-pseudokinase domain (Jak homology region 2) is important in the regulation of Jak2 kinase activity (44). Its presence is required for inhibition of Jak2 in the absence of cytokines and for cytokine inducibility of Jak2 activity and Stat5 phosphorylation. However, the exact mechanism of this regulation has not been elucidated. In conclusion, quantitative regulation of cytokine-

associated Jak2 may play a role in the Epo responsiveness of erythroid progenitors and may be controlled by distinct mechanisms dependent on either PKC or Btk.

Association of Btk with the TRAILR1 May Regulate Sensitivity to TRAIL-induced Apoptosis. Regulation of the apoptosis threshold is important during erythropoiesis and several apoptosis inducers have been implicated in erythropoiesis such as TNF α , FasL, or IFN γ (9, 45–47). In erythroid progenitors, caspase activation in absence of EpoR activation results in Gata-1 cleavage and apoptosis (48). The cytoplasmic serine kinase Raf is required to prevent caspase activation in immature cells (49). However, constitutive inhibition of caspases blocks erythroid differentiation (49) as caspase activity is required in late stages of erythroid differentiation to cleave proteins involved in nuclear integrity and chromatin condensation (50). We show that Btk can associate with the TRAILR1 in response to SCF, which may protect against TRAIL-induced apoptosis. Similarly, Btk has been shown to inhibit Fas-induced apoptosis in DT40 B cells, which was dependent on association of Btk with Fas (23). We suggest that Btk-mediated protection from apoptosis might involve a similar mechanism in both cell types (i.e., interaction of the respective death receptor with Btk). What remains puzzling is the observation that both Epo and SCF induce phosphorylation of Btk, whereas only SCF induces association of Btk with TRAILR1. This could be explained by spatial constraints, which would imply that the TRAILR1 and c-Kit are located in close proximity. More likely, the association of Btk with either the TRAILR1 or Fas requires an additional adaptor molecule that is regulated in response to SCF, but not in response to Epo. Such an adaptor molecule could also impose specificity of Btk for distinct death receptors, such as Fas or TRAILR1, in lymphoid or erythroid cells. In addition, the intact kinase domain of Btk is required for its association with Fas and probably also for its interaction with TRAILR1 (23). Because *wt* and Btk⁻ progenitors express similar levels of TRAILR1, the increased apoptosis induction in Btk erythroblasts cannot be due to different expression levels of death receptor.

Btk Deficiency Impairs Renewal and Enhances Differentiation at Limiting Cytokine Levels. Expansion of erythroid progenitors in the presence of Epo, SCF, and Dex is abrogated in Btk⁻ progenitors at limiting concentrations of Epo and SCF. Although the attenuated EpoR signaling and increased TRAIL sensitivity of Btk⁻ progenitors would have predicted increased apoptosis at limiting cytokine concentrations, we observed enhanced differentiation at the expense of renewal. Our recent results indicate that the role of EpoR signaling in early progenitors and maturing cells may be distinct in function. In early erythroblasts, signaling pathways (triggered via intracellular p-Tyr docking sites in the EpoR) contribute mostly to progenitor expansion because erythroblasts from mice expressing a respective truncated EpoR undergo normal differentiation, but are defective for expansion of erythroblasts (unpublished data). These pathways include Epo-induced phosphorylation of Ron/Gab1

and activation of PI3 kinase and MAPK (unpublished data). Furthermore, Epo-induced Stat5 activation is required for survival in erythroid differentiation (up-regulating Bcl-X_L), whereas lack of Stat5 in expanding progenitors causes differentiation at the expense of expansion in a Bcl-X_L-independent fashion (unpublished data). Moreover, because activated caspases contribute to execution of the late erythroid differentiation program where apoptosis is prevented by up-regulation of Bcl-X_L (49, 51), impaired protection against caspase activation in Btk⁻ cells could contribute to differentiation rather than to apoptosis. This suggests that the lack of Btk may enhance differentiation using both Epo- and SCF-activated signaling pathways.

Human X-linked agammaglobulinemia patients or Btk⁻ mice are not anemic and the mice do not present with increased spleen size (a hallmark for stress erythropoiesis) as do Stat5^{-/-} mice (52). Importantly, we analyzed Btk⁻ mice for their response to hypoxia, which was similar to *wt* littermates (see Supplemental Material). In vivo, Btk-deficient cells showed a disadvantage over *wt* cells very late in maturation (see Supplemental Material), but peripheral erythrocyte numbers and cell size were similar in *wt* and Btk⁻ animals. Accordingly, we also found no indications for increased Epo levels. Finally, numbers of early (BFU-E) and late (CFU-E) progenitors were similar in fetal livers of *wt* and Btk⁻ animals (see Supplemental Material).

In conclusion, Btk appears to be involved in the control of expansion, survival, and differentiation of erythroid progenitors via two distinct mechanisms: (a) via the regulation of Epo-induced signal transduction pathways with proliferation as the cellular readout and (b) via control of TRAIL-induced apoptosis, which may involve SCF-dependent association of Btk with TRAILR1. Interestingly, the combination of a reduced Epo response and enhanced sensitivity to death ligands is a known pathological condition and occurs, for example, in MDS (3, 8, 53). MDS constitutes a very heterogeneous hematologic disease group that occurs mainly in the elderly. Because a reduced response to Epo and death ligand sensitivity are common, this condition may be necessary but not sufficient to cause overt disease. Rather, additional genetic aberrations may be required for full development of MDS or even acute myeloid leukemia. Therefore, it will be interesting to examine whether Btk⁻ mice are prone to develop leukemia upon further genetic challenges (e.g., by retroviral insertion mutagenesis).

We want to thank Drs. T.B. van Dijk and I.P. Touw for many critical discussions regarding the work presented and Dr. J. Nimpf for support during the early phase of the work. We thank Dr. K. Stangl, G. Dingjan, S. Middendorp, and L. Felida for practical assistance, and Dr. J. Borst for the Btk^{wt} and Btk kinase dead cDNA constructs.

This work was supported by grants from the Dutch Cancer Society (EUR 1999–2064), the European Union (HPRN-CT-2000–00083), the START program (Y-163) of the FWF Austrian Research Fund to W. Ellmeier, and fellowships from the Dutch Academy of Arts and Sciences to M. von Lindern and from the Fonds zur Förderung der Wissenschaftlichen Forschung Austria (SFB006) to H. Beug.

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