Erythropoiesis in the absence of janus-kinase 2: BCR-ABL induces red cell formation in JAK2−/− hematopoietic progenitors

Saghi Ghaffari, Claire Kitidis, Mark D. Fleming, Hans Neubauer, Klaus Pfeffer, and Harvey F. Lodish

The receptor-associated protein tyrosine kinase janus-kinase 2 (JAK2) is essential for normal red cell development and for erythropoietin receptor (EpoR) signaling. JAK2−/− embryos are severely deficient in erythropoiesis and die at an early stage of development from fetal anemia. The binding of erythropoietin (Epo) to the EpoR triggers the activation of JAK2, the phosphorylation of the EpoR, and the initiation of the EpoR signaling cascade. In addition to Epo binding to its receptor, signaling pathways downstream of the EpoR can also be stimulated by the BCR-ABL oncoprotein. This study explored whether JAK2 is required for BCR-ABL-mediated stimulation of erythropoiesis. Here, it is shown that JAK2 is constitutively tyrosine phosphorylated in cultured and primary erythroid cells expressing BCR-ABL. However, BCR-ABL effectively supports normal erythroid proliferation, differentiation, and maturation in JAK2-deficient fetal liver cells. Using mutants of BCR-ABL, this study shows that certain signaling pathways activated by BCR-ABL segments distinct from its tyrosine kinase domain are essential for rescue of erythropoiesis in JAK2−/− progenitors. The consequences of these multiple signaling pathways for normal erythroid development are discussed.

Introduction

Erythropoietin (Epo) is essential for the proliferation, differentiation, and survival of erythroid cells as demonstrated definitively by the properties of Epo and erythropoietin receptor (EpoR)−/− mice.1−3 These mice die during embryogenesis from severe anemia, due to failure of erythroid colony-forming unit (CFU-E) progenitors to undergo terminal proliferation, differentiation, and red cell maturation. EpoR itself lacks any intrinsic catalytic activity; binding of Epo to the EpoR results in receptor dimerization and conformational changes, and phosphorylation and activation of receptor-associated JAK2.

Janus-kinase 2 (JAK2), a member of the JAK family of protein tyrosine kinases, is constitutively bound to cell surface EpoR and is crucial for EpoR signaling. JAK2−/− embryos die from profound anemia around day 11 to 13 of embryogenesis.5,6 Erythroid maturation is arrested earlier and more severely in JAK2−/− embryos compared with EpoR−/− embryos (Figure 1), because the absence of JAK2 abrogates the signaling not only of the EpoR but also of numerous additional cytokine receptors that are important for proliferation and survival of primitive erythroid progenitors.5,6 Activated JAK2 phosphorylates several tyrosine residues on the EpoR; these phosphotyrosine residues on the EpoR provide docking sites for SH2 (src-homology-2) domain-containing signal transduction proteins including STAT5 that bind to the receptor and are phosphorylated by JAK2, thereby initiating EpoR signaling cascades including activation of STAT5, Ras, mitogen-activated protein kinase (MAPK), JNK, P38, PI3-kinase–AKT, SHP1, SHP2, SHIP, and BCL-xL (reviewed in Constantinescu et al10). JAK2 may also directly phosphorylate and activate signaling proteins such as Shc in a receptor tyrosine-independent fashion; as another example, an EpoR devoid of any cytosolic tyrosines activates STAT5 to a level approximately 10% that of the wild-type receptor.9 The relative contribution of the different EpoR-activated signaling pathways to erythroid development, specifically proliferation, differentiation, and survival, of erythroid progenitors is not well understood.

JAK2 is the major JAK associated with and activated by a number of cytokine receptors besides the EpoR, including the growth hormone receptor, the type 2 interferon receptor, the thrombopoietin receptor Mpl, and the β common containing receptors that bind granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 3 (IL-3), IL-5, and IL-12 (reviewed in Yeh and Pellegrini10). Moreover, JAK2 is also associated with and activated along with other JAKs in response to the cardiotrophin receptor, the prolactin receptor, the granulocyte colony-stimulating factor receptor (G-CSF-R), and cytokine receptors containing gp130, such as IL-6R, ciliary neurotrophic factor-receptor (CNTF-R), leukemia-inhibitory factor-receptor (LIF-R), and oncostatin M-receptor (OSM-R) (reviewed in Yeh and Pellegrini10).

Many of the signaling pathways activated by JAK2 in response to Epo are also activated by the BCR-ABL oncoprotein (reviewed in Constantinescu et al12 and Ghaffari et al13), the molecular hallmark of chronic myeloid leukemia (CML). P210BCR-ABL (P210) and a related fusion protein P185BCR-ABL (P185) 12,13 are constitutively active protein tyrosine kinases whose activity is significantly more potent than their normal c-ABL counterpart.14,15 These
chimeric BCR-ABL proteins result from the fusion of the N-terminal segment of BCR (902 amino acids in P210 and 426 amino acids in P185) to most of the c-ABL protein. Despite a mild anemia which affects most patients at the chronic phase, erythroid progenitors are Epo-independent in culture and their number is increased in CML patients. In addition, BCR-ABL complements EpoR signaling and supports the proliferation, differentiation, and maturation of red cell progenitors when expressed in EpoR−/− fetal liver cells. Furthermore, BCR-ABL partially complements signals provided by IL-3, IL-6, and Steel factor (SF) required for proliferation and survival of primitive erythroid burst-forming unit (BFU-E) progenitors.

Since JAK2 is crucial for EpoR signaling and red cell development, we investigated whether JAK2 is required for BCR-ABL complementation of EpoR signaling. Although JAK2 was constitutively tyrosine phosphorylated in cultured and primary erythroid cells expressing BCR-ABL, erythropoiesis proceeded normally in JAK2−/− fetal liver cells transduced with BCR-ABL. This demonstrates that JAK2 is not required for BCR-ABL complementation of EpoR signaling.

In addition to the tyrosine kinase domain, several distinct sequences within BCR-ABL, either alone or in concert, contribute to BCR-ABL transforming potential by activating signaling pathways that are also activated by many cytokine receptors. Signal transduction pathways generated by BCR-ABL and its individual domains (Table 1) that promote proliferation and survival have been studied extensively both in vitro and in vivo. Thus, we also expressed several BCR-ABL mutants that exhibit impaired signaling (summarized in Table 1) in JAK2−/− fetal liver cells. Two of these mutants were defective in rescuing JAK2−/− progenitors but were able to rescue EpoR−/− progenitors. Thus, there exist signaling pathways activated directly by JAK2, independent of the EpoR (ie, in EpoR−/− cells), which are also activated by BCR-ABL and which are important for erythropoiesis. We discuss the potential importance of these findings for understanding of EpoR signaling.

**Materials and methods**

### Cells

Heterozygote JAK2−/− (129J × C57BL/6j background) mice were screened by polymerase chain reaction (PCR) as previously described, and bred and maintained at the Whitehead Institute animal facility. Individual E12.5 JAK2−/− fetal livers were isolated according to their distinct pale white color. They were dissected and disaggregated into single cell suspensions in alpha-modified minimum essential media (α-MEM, Stem Cell Technologies, Vancouver, BC, Canada) containing 15% fetal calf serum (FCS), passed through a 21-gauge needle, and washed 2 times in the same medium. A portion of the cells was diluted in 2% acetic acid to lyse mature erythrocytes and counted. Wild-type fetal liver cells were isolated from 14-day BALB/c embryos (Jackson Laboratories, Bar Harbor, ME).

The erythroleukemic cell line HCD57 was maintained in Iscoves modified Dulbecco medium (IMDM) containing 20% FCS, 0.01 M β-mercaptoethanol and erythropoietin (2 units/mL). HCD57 cells stably expressing P210 or the vector control were generated by electroporation of the murine stem cell virus (MSCV)-P210-pac or MSCV-pac retroviral vectors and selection of puromycin-resistant cells. To isolate Epo-independent HCDP210 cells, puromycin-resistant cells were cultured in the absence of Epo for 10 days and the resultant surviving cells were collected.

Phoenix packaging cells (kindly provided by Dr Gary Nolan, Stanford University, CA) were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% FCS.

### Retroviral constructs

cDNAs were cloned upstream of the internal ribosomal entry site (IRES) in the bicistronic retroviral MSCV-IRES-green fluorescent protein (GFP) vector, a gift of Dr Luk Van Parijs (Massachusetts Institute of Technology, Cambridge, MA). The translation of the complementary DNA (cDNA)−encoded protein and GFP is tightly linked in that the expression of GFP is proportional over a 100-fold range to the level of expression of the protein encoded by the cDNA placed upstream of the IRES. To construct the vectors MSCV-P210-IRES-GFP, MSCV-P210 Y177F-IRES-GFP, MSCV-P210 ΔSH2-IRES-GFP, MSCV-P185-IRES-GFP, and MSCV-P185 Δ176-427-IRES-GFP, the desired inserts were flanked by EcoRI sites and cloned into the corresponding site in the MIG vector. The BCR-ABL P185 triple mutant (P185 TM) and JAK2 cDNA were inserted by blunt-end ligation into the HpaI site of the MIG vector. MSCV-P210-pac was described previously.

### Retroviral supernatant production and infection procedure

High titer replication-free retroviral supernatants were generated as follows: 10 μg retroviral plasmids together with the pCL-Eco vector were cotransfected using calcium phosphate (Invitrogen kit) into 106 cells Phoenix packaging cells plated on 60-mm dishes 18 hours prior to

<table>
<thead>
<tr>
<th>BCR-ABL mutant</th>
<th>Signaling protein binding/ pathways abrogated</th>
<th>Growth factor independence of hematopoietic cells</th>
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<th>CML-like disease in mice</th>
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<tr>
<td>P210ΔSH2</td>
<td>Tyrosine phosphorylated proteins, PI3-kinase/AKT(1)</td>
<td>0.30±0.00</td>
<td>_5.85,56</td>
<td>5.3,54</td>
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<td>P210 Y177F</td>
<td>Grb-2/Sos/Ras(1)</td>
<td>0.30±0.01</td>
<td>_3.1</td>
<td>5.9</td>
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<tr>
<td>P185 ΔSH2</td>
<td>Grb-2/Sos/Ras(1, 2)</td>
<td>0.30±0.01</td>
<td>_3.1,3.2</td>
<td>ND</td>
</tr>
<tr>
<td>P185 TM†</td>
<td>tyrosine phosphorylated proteins</td>
<td>0.30±0.01</td>
<td>_3.1</td>
<td>ND</td>
</tr>
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*In vitro transformation by individual mutants assessed by suppression of contact-dependent growth in a Rat-1 focus formation assay, abrogation of anchorage-dependent growth in a Rat-1 soft agar colony formation assay, or propagation of lymphoid cells in long-term bone marrow cultures.

†P185 TM contains a mutation in the Grb-2 binding site (Y177F), the phosphotyrosine-binding site within the SH2 domain (R552L), and an autophosphorylation site (Y793F).
transfection. The resulting retroviral supernatant was collected 48 hours later and was used to infect fetal liver cells. Titers of $2 \times 10^9$ to $8 \times 10^9$ were routinely obtained. A 1-t0-5 dilution of the packaged virus was used to infect NIH 3T3 cells and protein expression was analyzed by Western blot using the appropriate antibody (anti-ABL or anti-JAK2 antibodies). JAK2 ~ fetal liver cells (2 $\times 10^6$ cells/mL) were resuspended in viral supernatants at a multiplicity of infection of 5 to 10 and plated in 60 mm rectoronic (Takara Biomedicals)-coated dishes in the presence of 100 ng/mL each of IL-6 and SF (PeproTech) for 36 hours. Cells were then washed once and resuspended in α-MEM containing 15% FCS and the same growth factors for another 24 hours. Wild-type fetal liver cells were incubated with IL-3 (6 ng/mL) (PeproTech), IL-6 (10 ng/mL), and SF (100 ng/mL) for 24 hours prior to infection. Cells were resuspended in the appropriate viral supernatant in the presence of the same growth factors for 48 hours. Cells were then washed and resuspended in media containing the same growth factors for an additional 24 hours.

Flow cytometry and immunostaining
Retrovirally infected cells were washed twice in phosphate buffer saline (PBS) solution containing 2% FCS. Cells were then incubated with control rat serum at room temperature for 15 minutes, followed by incubation with 1 µg/mL Ter119-PE antibody (BD PharMingen) for 30 minutes on ice. Afterward, the cells were washed once with cold PBS 2% FCS, and once with PBS 2% FCS containing 1 µg/mL propidium iodide (PI), resuspended in 500 µL PBS containing 2% FCS prior to a FACS sort (Becton Dickinson). GFP or GFP+ Ter119- cells were selected and FACS-sorted for further analysis by cytospin or colony assays. CELLQuest (Becton Dickinson) was used for FACS analysis.

Colony assays
Retrovirally transduced cells were washed once in α-MEM containing 15% FCS and plated in duplicate in semisolid medium containing 0.9% g/mL bovine serum albumine (BSA), 10 µg/mL bovine insulin, 200 µg/mL human transferrin, 10⁻⁴ M 2-mercaptoethanol, 2 mM L-glutamine (MethoCult M3234; StemCell Technologies), to measure colony formation as previously described.¹⁸,²³ CFU-E formation was carried in methylcellulose cultures containing SF (100 ng/mL; PeproTech) with or without Epo (3 units/mL; Amgen, Thousand Oaks, CA), while BFU-E formation was assayed in methylcellulose cultures in the presence or absence of IL-6 (10 ng/mL) and SF (100 ng/mL), with or without Epo (3 u/mL). The number of CFU-E colonies was determined after dexamethasone staining of hemoglobin and counted 2 days after plating. BFU-E colonies of hemoglobinized erythroblasts were counted after 9 days. Colonies were individually aspirated for reverse transcriptase (RT-PCR) or cytospin, and analysis of their erythroblast morphology determined after Wright Giemsa staining.

Cytospin and cytoplasmic staining
Cells were washed in PBS with 2% FCS, and resuspended in PBS containing 1% BSA at a concentration of $3 \times 10^5$ cells/mL. Cells (100 µL per slide) were subjected to a cytospin for 2 minutes at 600 rpm (Cytospin 3; Shandon) and air dried. Cells were then stained with Wright Giemsa (Harlec) according to the manufacturer’s recommendations.

PolyA RT-PCR from single erythroid colonies
To analyze the gene expression profile of transduced erythroid colonies, we performed RT-PCR using oligo-dT-based primers and a polyA tailing strategy as previously described.²⁴,²⁵ Briefly, single BFU-E colonies were aspirated (2 µL to 10 µL) from methylcellulose plates and lysed directly in a 5 M guanidinium isothiocyanate solution containing 20 mM dithiothreitol. Nucleic acids were precipitated and the entire sample was reverse transcribed using an oligo-dT primer (1 µg/mL) (5’-CAT-GTC-GTC-CAG-GCC-GCT-CTG-GAC-AAA-ATA-TGA-ATT-C[T]₆-3’), tailed, and subjected to PCR²⁵ containing the oligo-dT primer described above. Total cDNA was amplified using 5 units of Taq polymerase. One-fourth of the total amplified product from each colony was separated by electrophoresis through 1% agarose and transferred to a Zeta-probe GT membrane (Bio-rad) and probed with either β-major globin,²⁶ GATA-1,²⁴,²⁷ or L-32.²⁴ Probes (Figure 3D) were prepared as previously described.²⁴,²⁷

Immunoprecipitation and Western blot analysis
Populations of HCD57 or HCDP210 cells or of infected wild-type fetal liver cells were mixed 4 times in serum-free media and starved overnight in IMDM containing 0.1% FCS. HCD57 and HCDP210 (5 × 10⁶ cells), and fetal liver cells (2 × 10⁶ cells) were stimulated 18 hours later with or without Epo (100 u/mL) for 5 minutes at 37°C. Cells were then washed twice with cold PBS and extracts were prepared by the addition of 1 mL lysis buffer, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 2 mM Na₂VO₄, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1% Brij-96, 1 mM diithiothreitol (DTT). For immunoprecipitation, cell extracts were incubated overnight at 4°C with 5 µL anti-JAK2 polyclonal anti-sera (Upstate Biotechnology, Lake Placid, NY) or with 1 µg mouse anti-c-ABL monoclonal antibody (sc-23; Santa Cruz). Immunocomplexes were recovered by binding to protein A-Sepharose or protein G-Sepharose beads (Roche-Boehringer). The captured immunocomplexes were washed 3 times with lysis buffer and once with PBS and were then eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer. Samples were fractionated through SDS-polyacrylamide gels, transferred electrophoretically to nitrocellulose membranes, and incubated with the indicated antisera: (1) anti-phosphotyrosine monoclonal antibodies (4G10, Upstate Biotechnology) (1:1000); (2) anti-JAK2 polyclonal antibodies (1:1000); and (3) anti-ABL monoclonal antibodies (1:1000). Bound antibodies were detected by enhanced chemiluminescence system (DuPont-NEN).

Results
JAK2 is constitutively phosphorylated in cultured erythroleukemia cells and primary erythroid cells expressing BCR-ABL
We first examined the tyrosine phosphorylation status of JAK2 in an erythroid cell line expressing BCR-ABL. We expressed P210 in the Epo-dependent erythroleukemic HCD57 cells (Figure 2A) and derived a population that proliferated in the absence of Epo (HCDP210 cells, Figure 2B). The Western blot in Figure 2A shows that P210 is expressed in the Epo-independent HCDP210 cells (right panel) and as expected, P210 BCR-ABL but not c-ABL (left panel) was tyrosine phosphorylated in HCDP210 cells starved of all growth factors. As shown in Figure 2C. JAK2 is tyrosine phosphorylated and thus activated in the presence but not the absence of Epo in HCD57 cells. In contrast, HCDP210 cells exhibited a low level of JAK2 tyrosine phosphorylation in the absence of Epo, and Epo stimulation induced a further increase of JAK2 tyrosine phosphorylation (Figure 2C, upper panel). In HCDP210 cells, Epo addition also caused the mobility of the JAK2 tyrosine phosphorylation (Figure 2C, upper panel) was tyrosine phosphorylated in HCDP210 cells starved of all growth factors. As shown in Figure 2C. JAK2 is tyrosine phosphorylated and thus activated in the presence but not the absence of Epo in HCD57 cells. In contrast, HCDP210 cells exhibited a low level of JAK2 tyrosine phosphorylation in the absence of Epo, and Epo stimulation induced a further increase of JAK2 tyrosine phosphorylation (Figure 2C, upper panel). In HCDP210 cells, Epo addition also caused the mobility of the

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of both GFP and P210 in the same cell (see “Materials and methods”).

Wild-type E14 fetal liver cells were retrovirally transduced with either MSCV-P210-IRES-GFP or the control vector and analyzed by FACS for GFP expression. Approximately 50% of P210-infected and 70% of control vector–infected fetal liver cells expressed the GFP marker (Figure 3A). To avoid cellular loss, we subjected the totality of these populations, without further FACS selection, to serum starvation and examined the tyrosine phosphorylation status of JAK2 in response to Epo stimulation. A low level of JAK2 tyrosine phosphorylation in response to Epo stimulation was detected in primary fetal liver cells infected with control vector but, as expected, none was detected in the absence of Epo (Figure 3B, upper panel). In contrast, tyrosine phosphorylation of JAK2 was easily detectable in P210-transduced fetal liver cells starved in the absence of serum and Epo, and Epo stimulation did not affect the level of JAK2 tyrosine phosphorylation (Figure 3B, upper panel, i). A shorter exposure of the Western blot confirmed the conclusion that the level of JAK2 tyrosine phosphorylation in BCR-ABL–transduced cells is unaffected by addition of Epo (Figure 3B, upper panel, ii). As a control, we showed that JAK2 protein was present at equal levels in P210-transduced and control fetal liver cells (Figure 3B, lower panel).

Thus, JAK2 becomes constitutively tyrosine phosphorylated and presumably active, as a result of BCR-ABL expression both in HCD57 erythroleukemia and in fetal liver erythroid cells.

JAK2 is not required for red cell formation by BCR-ABL

To assess whether tyrosine phosphorylation of JAK2 and activation of JAK2 signaling pathways is required for BCR-ABL–induced maturation of erythroid progenitors, we examined the potential of P210 to support red cell formation in the absence of JAK2 by using primary JAK2−/− fetal liver cells. To this end, E12.5 JAK2−/− fetal liver cells were retrovirally transduced with either P210, JAK2, or vector control and analyzed for GFP expression as a measure of infection efficiency (Figure 4B; see “Materials and methods”). We established optimum conditions (Figure 4A) for retroviral transduction of JAK2−/− fetal liver cells; this also allowed for a significant expansion of transduced erythroid progenitors. Furthermore, this system provided reproducible conditions for direct comparison of the effects of transduced P210 and JAK2 on JAK2−/− fetal liver cells.

Ter119 is a marker of erythroid differentiation expressed on the cell surface of most (80%-90%) wild-type E14 fetal liver cells (data not shown). In contrast, Ter119 is present only on 10% to 20% of JAK2−/− fetal liver cells, consistent with the notion that these cells are blocked in an early stage of erythroid differentiation (and data not shown). We therefore used Ter119 as a marker of erythroid differentiation and analyzed its expression two and a half days postinfection by flow cytometry of retrovirally transduced GFP-positive cells. In contrast to control-infected cells, which exhibited...
less than 20% Ter119 positivity within the GFP-positive population, these “Ter119 positive cells” clearly include many autofluorescent cells within the chosen window (Figure 4B, bottom panel), in P210- and JAK2-infected populations 30% to 50% of GFP-positive cells were Ter119-positive (Figure 4B). Thus, P210 expression induced a significant proportion of Ter119-positive cells even in the absence of JAK2.

To confirm this key point, we used the FACS to isolate all retrovirally transduced (ie, GFP-positive) cells from JAK2 and P210-infected populations, regardless of their Ter119 expression.
JAK2-transduced colonies derived from JAK2 by the hatched bars. Graphs are averages from duplicate cultures of 4 (A) and 5 (B) independent experiments. Under optimum conditions, the average absolute number of colonies formed in the JAK2-infected populations and cultured under optimum conditions (Epo and SF for CFUE [A] and Epo, SF, and IL-6 for BFU-E [B]) shown cultured in the presence or absence of 2 u/mL Epo, 100 ng/mL SF, and 10 ng/mL IL-6 (B) were counted after 2 (A) and 9 (B) days, respectively. Results are presented as percentage of colonies formed in the JAK2-infected populations and cultured under optimum conditions (Epo and SF for CFUE [A] and Epo, SF, and IL-6 for BFU-E [B]) shown by the hatched bars. Graphs are averages from duplicate cultures of 4 (A) and 5 (B) independent experiments. Under optimum conditions, the average absolute number of JAK2-transduced colonies derived from JAK2 fetal liver cells is 1248 ± 263 CFU-E and 96 ± 9 BFU-E colonies per 10^7 GFP+ JAK2 FACS-sorted cells. (C) Representative BFU-E-derived colonies from GFP+ JAK2+ fetal liver cells transduced with either P210 or JAK2 and cultured in the presence of SF and IL-6, with (JAK2-transduced cells) or without (P210-transduced cells) or without (P210-transduced cells) Epo (× 100). (D) PolyA RT-PCR from single P210- or JAK2-generated BFU-E colonies of similar size were analyzed by southern blotting probed with β-major globin, GATA-1, and L-32. Shown are representative results from 3 independent experiments. Controls consist of HCD57 (10^5 cells) in lane 1 and PCR reagents with no cells in lane 2.

Figure 5. BCR-ABL rescues JAK2−/− erythroid progenitors. The population of GFP+ Ter119+ cells encompasses all retrovirally transduced erythroid progenitors and, as detailed in the legend to Figure 4B, this population was FACS sorted from JAK2−/− fetal liver cells transduced with either JAK2 or P210 BCR-ABL. From these, CFU-E (A) and BFU-E (B) colonies were generated. Total numbers of diaminobenzidine-positive CFU-Es cultured in the presence or absence of 2 u/mL Epo and 100 ng/mL SF (A) and BFU-Es cultured in the presence or absence of 2 u/mL Epo, 100 ng/mL SF, and 10 ng/mL IL-6 (B) were counted after 2 (A) and 9 (B) days, respectively. Results are presented as percentage of colonies formed in the JAK2-infected populations and cultured under optimum conditions (Epo and SF for CFUE [A] and Epo, SF, and IL-6 for BFU-E [B]) shown by the hatched bars. Graphs are averages from duplicate cultures of 4 (A) and 5 (B) independent experiments. Under optimum conditions, the average absolute number of JAK2-transduced colonies derived from JAK2−/− fetal liver cells is 1248 ± 263 CFU-E and 96 ± 9 BFU-E colonies per 10^7 GFP+ JAK2 FACS-sorted cells. (C) Representative BFU-E-derived colonies from GFP+ JAK2+ fetal liver cells transduced with either P210 or JAK2 and cultured in the presence of SF and IL-6, with (JAK2-transduced cells) or without (P210-transduced cells) Epo (× 100). (D) PolyA RT-PCR from single P210- or JAK2-generated BFU-E colonies of similar size were analyzed by southern blotting probed with β-major globin, GATA-1, and L-32. Shown are representative results from 3 independent experiments. Controls consist of HCD57 (10^5 cells) in lane 1 and PCR reagents with no cells in lane 2.

(Figure 4B, populations i and ii) and analyzed their morphology (Figure 4C). A majority of GFP-positive cells transduced with P210 were morphologically erythroid, at different stages of differentiation, as were a majority of JAK2-transduced cells that were cultured in the presence of Epo (Figure 4C). In contrast, freshly isolated JAK2−/− fetal liver cells, prior to infection, exhibited only a few erythroid cells (Figure 4D). Specifically, expression either of JAK2 (in the presence of Epo) or P210 (in the absence of Epo) induced terminal erythroid differentiation and maturation of some of JAK2−/− fetal liver cells (Figure 4Ci,ii,iii). In contrast, freshly isolated JAK2−/− fetal liver cells did not exhibit any mature enucleated erythrocytes. Interestingly, JAK2-transduced cells cultured in the presence of Epo were found mostly at a slightly later stage of differentiation (60% orthochromatropic erythroblasts and 33% polychromatophilic erythroblasts) as compared with P210-transduced cells (which contained only 25% orthochromatropic erythroblasts and 58% polychromatophilic erythroblasts). Thus, JAK2 and BCR-ABL are similar, but not identical, in their abilities to support induction of erythroid differentiation and maturation from JAK2−/− fetal liver cells.

Ter119 positive cells are composed of erythroblasts at different stages of differentiation and do not give rise to BFU-E or CFU-E erythroid colonies (data not shown). Thus, they do not contain any committed erythroid progenitors. Consequently, the population of GFP+ Ter119+ cells encompasses all retrovirally transduced cells including all transduced progenitors of erythroid and other hematopoietic cell type origin. Thus, GFP+ Ter119+ cells, the population relatively enriched in transduced erythroid progenitor cells, were selectively FACS-sorted from infected JAK2−/− fetal liver cells (population ii in Figure 4B) and plated in semisolid cultures in the presence of SF. CFU-E-derived colonies that stained positively with diaminobenzidine, a hemoglobin marker, were counted 2 days later. JAK2−/− fetal liver cells transduced with P210 generated as many mature erythroid CFU-E progenitors in vitro as did cells transduced with JAK2 and cultured in the presence of Epo (Figure 5A). The size of clusters and the degree of hemoglobinization were comparable between the 2 groups (data not shown). No CFU-E colonies were formed from JAK2−/− fetal liver cells infected with the control vector (Figure 5A).

Similarly, P210 expression in JAK2−/− fetal liver cells, cultured in the presence of SF and IL-6 but in the absence of Epo, induced the generation of primitive BFU-E-derived colonies (Figure 5B). P210-induced BFU-E colonies were similar in number to those induced by the expression of JAK2 in JAK2−/− fetal liver cells and then cultured with Epo in addition to SF and IL-6. P210- and JAK2-generated BFU-E colonies were comparable in size, morphology, and degree of hemoglobinization (Figure 5C), and contained morphologically normal erythroblasts (data not shown). In addition, these colonies expressed erythroid genes such as β-major globin and GATA-1 (Figure 5D). Taken together, these data demonstrate that P210-generated BFU-E colonies from JAK2−/− fetal liver cells were in all aspects similar to JAK2-rescued erythroid colonies cultured with Epo. Thus, P210 and JAK2 are equivalent in their abilities to support BFU-E and CFU-E formation in fetal liver cells lacking JAK2.

In addition to Epo, for their optimum proliferation and survival in in vitro culture assays, primitive erythroid progenitors require cytokines and growth factors such as IL-3, IL-6, GM-CSF, and SF. Previously, we showed that P210-transduced EpoR−/− fetal liver cells generate a significant numbers of...
Results are obtained in the absence of Epo and are shown as a percentage of control ± se; the 100% control is determined by (1) expression of P185 in wild-type fetal liver resulting in generation of 5230 ± 1050 CFU-E per 10⁶ GFP Ter119⁻ FACS-sorted cells or (2) expression of P210 in JAK2⁻⁻ fetal liver resulting in generation of 1268 ± 221 per 10⁶ GFP Ter119⁻ FACS-sorted cells.

*P < .001.
†P < .01.

The P value for each mutant is determined by comparing numbers obtained for the mutant to the ones obtained for P185, in wild-type fetal liver cells, and to the numbers obtained for P210, in JAK2⁻⁻ fetal liver cells. ND indicates not determined.

Discussion

Cumulative evidence in the past decade supports the notion that cell fate is determined by the cellular context in which signaling proteins are expressed. Tyrosine phosphorylation and activation of JAK2 is key to erythropoietic development. In response to Epo stimulation, JAK2 becomes tyrosine phosphorylated and activated; JAK2 then phosphorylates the EpoR on several tyrosine residues leading to the activation of multiple signaling pathways [reviewed in Constantinescu et al⁷]. In addition to the EpoR, JAK2 binds to other cytokine receptors and cytosolic proteins, and directly activates, in a receptor-phosphotyrosine independent fashion, signaling pathways such as STAT5 and Shc.⁸,⁹ The relative contribution of different EpoR-activated signaling pathways to erythroid development, specifically proliferation, differentiation, and survival of erythroid progenitors, is not well understood. As a first step in identifying potential EpoR signaling components sufficient to support erythropoiesis in primary erythroid cells, we activated signaling pathways downstream of EpoR that overlap with signaling pathways activated specifically by BCR-ABL amino acids 176-247.

Figure 6. Comparable protein expression in cells transduced with BCR-ABL mutant retroviral supernatants. Western blot analysis of expression of BCR-ABL and its mutant in ST3 cells infected with a 1-in-10 dilution of transducing retroviral particles used in experiments summarized in Table 2. The arrow indicates the endogenous c-ABL protein.
The constitutively active protein tyrosine kinase BCR-ABL supports erythropoiesis in both wild-type and EpoR--/-- fetal liver cells.18 In BCR-ABL-expressing erythroid cells, JAK2 is tyrosine phosphorylated (Figures 2 and 3). Thus, we asked whether JAK2 is an obligatory signaling protein for induction of erythropoiesis downstream of BCR-ABL. Using Ter119 as a cell surface marker of erythroid cells, functional progenitor assays, and morphologic analyses, we have demonstrated that JAK2 is not required for BCR-ABL induction of red cell formation. However, our data indicate that BCR-ABL does not support erythroid maturation quantitatively as well as JAK2 does and that these two protein tyrosine kinases are not identical in their ability to support terminal differentiation and maturation of erythroid cells.

Our findings also indicate that the ability of BCR-ABL to activate signaling pathways required for erythropoiesis is different in EpoR--/-- and JAK2+/-- fetal liver cells. As example, BCR-ABL expression in EpoR--/-- fetal liver cells supports the formation of 25% of the maximum number of BFU-E-derived colonies in the absence of any added growth factors, namely IL-3, IL-6, and SF.18 In contrast, here we showed that in the absence of JAK2, BCR-ABL expression did not alleviate the requirement for SF and IL-6, in generation of BFU-E-derived erythroid colonies.

Other examples are provided by the BCR-ABL mutants lacking either the SH2 domain (P210 ΔSH2), or the Y177 residue that is essential for activation of the Ras pathway by BCR-ABL in certain cells. Despite their impaired signaling potential, P210 ΔSH2 or P210Y177F replace EpoR signaling and support erythropoiesis in EpoR--/-- fetal liver cells to the same extent as does P210.18 In contrast, here we found that P210 ΔSH2 and P210Y177F do not support efficient erythroid development in the absence of JAK2 (Table 2). Taken together, these results suggest that tyrosine phosphorylation and presumably activation of JAK2 in BCR-ABL-expressing cells activates signaling pathways that overlap with those activated by the P210 SH2 domain or P210 (phospho) Y177. Activation of these pathways either via JAK2 or these BCR-ABL domains is important for normal erythropoiesis in EpoR--/-- and also in wild-type fetal liver cells.

Although JAK2 is constitutively tyrosine phosphorylated in BCR-ABL-expressing erythroid (Figures 2, 3) and other myeloid cells,33,34 the mechanism of this effect is unknown. Interestingly, JAK2 immunocomplexes recovered from HCDP210 cells constantly contained P210 (data not shown), suggesting P210 and JAK2 are interacting in BCR-ABL-expressing erythroid cells and that BCR-ABL may directly phosphorylate JAK2. The tyrosine residues in JAK2 that become phosphorylated in BCR-ABL-expressing cells are unknown. Phosphorylation of certain tyrosines in JAK2 may directly activate its kinase activity. Alternatively, phosphotyrosines can provide docking sites for binding SH2 or phosphotyrosine binding domains of cytosolic proteins that subsequently become phosphorylated by JAK2.

In this study we identified at least one domain of BCR-ABL that is crucial for erythroid development in both wild-type and JAK2+/-- fetal liver cells. The 176-427 deletion mutant of P185 (P185 Δ176-427) did not support erythropoiesis in the absence of Epo when expressed in fetal liver cells, regardless of whether they expressed JAK2. For several reasons the failure of P185 Δ176-427 to support wild-type fetal erythropoiesis in the absence of Epo does not appear to be due to the absence of the Grb-2-binding site Y177; first, we showed previously that Y177 is dispensable for BCR-ABL complementation of EpoR signaling in EpoR--/-- fetal liver cells.18 Second, the Y177 residue is also missing in P185 TM, which we showed here supports wild-type fetal liver erythropoiesis better than does P185 Δ176-427 (Table 2). Moreover, despite the lower potency of P185 Δ176-427 as compared with P185 TM in its ability to support erythropoiesis, P185 Δ176-427 is more potent than P185 TM in its transforming ability.30 The failure of the P185 Δ176-427 mutant to support erythropoiesis in fetal liver cells is likely not due to a lack of STAT5 activation, since the BCR-ABL kinase domain and domains supporting the activation of STAT523 are all conserved in this mutant.

Some clues to the deficient support of erythropoiesis by the P185 Δ176-427 mutant may come from an analysis of its missing sequence: sequences within 176-427 of BCR bind BCR-associated protein-1, a member of the 14-3-3 family of adapter proteins.36 The 14-3-3 family of proteins binds to serine/threonine phosphorylated proteins such as the MAPKKK Raf137,38, the proapoptotic protein BAD, a BCL-2 family member; and the transcription factor Forkhead, which increases the expression of apoptotic genes39,40 all targets of the serine threonine kinase AKT.41 Upon phosphorylation by activated AKT, Forkhead and BAD bind 14-3-3 proteins and are subsequently sequestered in the cytoplasm away from their targets, thereby inhibiting their proapoptotic functions.41 Interestingly, the 176-427 sequence within BCR-ABL has been shown to regulate PI3-kinase/AKT,42 Raf1,43 and the 14-3-3 family of adapter proteins,46 all implicated in erythropoiesis44-46 (and S.G. and H.L., unpublished data, August 2000). Our recent findings support an important role for AKT in erythroid development.47 In addition, the data presented here may point to a central role for the 14-3-3 family of adapter proteins in erythropoiesis and we are currently investigating this possibility.

STAT5 activation is important for erythroid survival as demonstrated by studies of STAT5b/-- fetal liver mice.48 BCR-ABL expression in hematopoietic cultured cells line clearly results in the activation of STAT55,34 and its known transcriptional target BCL-xL.49 However, the role of STAT5 in BCR-ABL function is not clear.50-52 STAT5 is moderately phosphorylated in BCR-ABL-expressing myeloid cells isolated from mice with CML-like disease.53,54 Moreover, STAT5 was not activated in Epo-independent HCDP210 cells as assessed by electrophoretic mobility shift assay (data not shown). The SH2 and SH3 domains of BCR-ABL have been reported to be required together for activation of the STAT5 pathway.55 Although it contains intact SH2 and SH3 domains, we found that P185 Δ176-427 was unable to support fetal liver erythropoiesis in the absence of Epo. Furthermore, STAT5b/-- fetal liver cells transduced with BCR-ABL generated normal numbers of erythroid colonies in the absence of Epo (S.G. and H.L., unpublished data, February 2000), and thus we exclude any significant role for STAT5 in BCR-ABL complementation of EpoR signaling. Taken together, our findings support the notion that pathways other than STAT5, potentially involving 14-3-3, PI3-kinase/AKT, and/or Raf-1 are activated by BCR-ABL in fetal liver cells and are essential for normal erythropoiesis.
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References

11. Gaffney S, Daley QG, Lodish HF. Growth factor independence and BCR/ABL transformation: promise and pitfalls of murine model systems and critical reading of the manuscript and to Glenn Paradis (MIT, Cancer Center) for cell sorting. We are indebted to Guy Sauvageau and his group (Clinical Research Institute of Montreal) for providing invaluable help with single cell polya-RT-PCR.

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Erythropoiesis in the absence of janus-kinase 2: BCR-ABL induces red cell formation in JAK2−/− hematopoietic progenitors

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