JOURNAL OF VIROLOGY, June 2006, p. 5678–5685 Vol. 80, No. 12
0022-538X/06/$08.00+0 doi:10.1128/JVI.02651-05

Erythroblast Transformation by the Friend Spleen Focus-Forming Virus Is Associated with a Block in Erythropoietin-Induced STAT1 Phosphorylation and DNA Binding and Correlates with High Expression of the Hematopoietic Phosphatase SHP-1

Kazuo Nishigaki,† Charlotte Hanson, Takashi Ohashi,‡ Angelo Spadaccini,§ and Sandra Ruscetti*

Laboratory of Cancer Prevention, National Cancer Institute—Frederick, Frederick, Maryland

Received 19 December 2005/Accepted 23 March 2006

Infection of mice with Friend spleen focus-forming virus (SFFV) results in a multistage erythroleukemia. In the first stage, the SFFV envelope glycoprotein interacts with the erythropoietin receptor and a short form of the receptor tyrosine kinase sf-Stk, resulting in constitutive activation of signal transducing molecules and the development of erythropoietin (Epo)-independent erythroid hyperplasia and polycythemia. The second stage results from the outgrowth of a rare virus-infected erythroid cell that expresses nonphysiological levels of the myeloid transcription factor PU.1. These cells exhibit a differentiation block and can be grown as murine erythroleukemia (MEL) cell lines. In this study, we examined SFFV MEL cells to determine whether their transformed phenotype was associated with a block in the activation of any Epo signal-transducing molecules. Our studies indicate that Epo- or SFFV-induced activation of STAT1/3 DNA binding activity is blocked in SFFV MEL cells. The block is at the level of tyrosine phosphorylation of STAT1, although Jak2 phosphorylation is not blocked in these cells. In contrast to Epo, alpha interferon can induce STAT1 phosphorylation and DNA binding in SFFV MEL cells. The SFFV-transformed cells were shown to express elevated levels of the hematopoietic phosphatase SHP-1, and treatment of the cells with a phosphatase inhibitor restored STAT1 tyrosine phosphorylation. MEL cells derived from Friend murine leukemia virus (MuLV) or ME26 MuLV-infected mice, which do not express PU.1, express lower levels of SHP-1 and are not blocked in STAT1/3 DNA-binding activity. Our studies suggest that SFFV-infected erythroid cells become transformed when differentiation signals activated by STAT1/3 are blocked due to high SHP-1 levels induced by inappropriate expression of the PU.1 protein.

The Friend spleen focus-forming virus (SFFV) is a highly pathogenic retrovirus that induces erythroleukemia in susceptible strains of mice (47). Friend SFFV, a replication-defective retrovirus, carries a unique *env* gene encoding a 55-kDa glycoprotein which is responsible for its pathogenicity. The first stage of the disease induced by the polycythemic strain of Friend SFFV (SFFV-P) is characterized by splenomegaly and polycythemia and is due to the polyclonal expansion and differentiation of erythroid cells in the absence of the erythroid hormone erythropoietin (Epo). This Epo-independent erythroblastosis is due to the cell surface interaction of the SFFV envelope protein with the erythropoietin receptor (EpoR) and a short form of the receptor tyrosine kinase Stk (sf-Stk) (3, 12, 22, 35). The second stage of the disease consists of the outgrowth of Friend SFFV-infected erythroid cells that have become transformed due to integration of the virus into the *Sfpi-1* locus (29, 39, 40). This leads to inappropriate expression of the *Sfpi-1* gene product, PU.1, in erythroid cells, causing a block in their differentiation and the outgrowth of transformed erythroleukemia cells that can be grown as murine erythroleukemia (MEL) cell lines (50).

The Epo-independent erythroid hyperplasia and polycythemia observed in the first stage of the disease induced by Friend SFFV-P results from the constitutive activation of signals from the EpoR due to its interaction with SFFV gp55 and sf-Stk. Components of most of the signal transduction pathways induced in erythroid cells by Epo, including the Jak-STAT pathway (30, 31, 34, 37), are constitutively activated in SFFV-infected cells in the absence of Epo. It is not known which of these signals are required for erythroid cell differentiation, but studies with a variant of SFFV that can induce Epo-independent erythroid hyperplasia but not polycythemia suggest that activation of STAT proteins by SFFV gp55 may be involved (S. Ruscetti, unpublished data). Previous studies have shown that both Epo and SFFV can induce STAT 1, 3, and 5 DNA binding activity in erythroid cells (7, 14, 15, 19, 20, 37, 41, 43). Since erythroid cells transformed by SFFV in the second stage of the disease are blocked in differentiation, we carried out studies to determine if either Epo or SFFV could still activate STAT proteins in these cells. Our studies indicate that activation of STAT1/3 DNA binding activity by either Epo or SFFV, but not by alpha interferon (IFN-α), is blocked in transformed MEL cell lines from SFFV-infected mice. This block in STAT DNA binding activity, which is directly correlated with expression of

* Corresponding author. Mailing address: Laboratory of Cancer Prevention, Building 469, Room 205, National Cancer Institute—Frederick, Frederick, MD 21702-1201. Phone: (301) 846-5740. Fax: (301) 846-6164. E-mail: ruscetti@ncifcrf.gov.

† Present address: Department of Immunotherapeutics, Tokyo Medical and Dental University, Tokyo 113-8519, Japan.

‡ Present address: Institute for Genetic Medicine, Hokkaido University, Sapporo 060-0815, Japan.

§ Present address: Office of Vaccines Research and Review, Division of Viral Products, Center for Biologics Evaluation and Research, Bethesda, MD 20892.

the PU.1 protein, is associated with a block in phosphorylation of STAT1 and expression of high levels of the hematopoietic phosphatase SHP-1.

MATERIALS AND METHODS

Cell lines and primary erythroleukemia cells. The erythroleukemia cell lines DS19, C19, NP1, NP4, NP5, NP7, and NP13 (54) were established from mice infected with Friend SFFV and were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5×10^{-5} M 2-mercaptoethanol (2-ME). The Epo-dependent erythroleukemia cell line HCD-57, which was derived from an NIH Swiss mouse infected with Friend murine leukemia virus (MuLV) (48), was maintained in Iscove's modified Dulbecco minimal essential medium (IMDM) supplemented with 30% FCS, 5×10^{-5} M 2-ME, and 2 U/ml of Epo. TP3, HB22.2, and CB7 (1, 38, 52), Epo-independent erythroleukemia cells lines derived from mice infected with Friend MuLV, were maintained in DMEM supplemented with 10% FCS and 5×10^{-5} M 2-ME. SR15, SR7, SR40, and SR54 (46), which are Epo-dependent hematopoietic cell lines established from mice injected with the *gag-myb-ets*-containing ME26 virus (62), were maintained in IMDM supplemented with 30% FCS, 5×10^{-5} M 2-ME, and 2 U/ml of Epo.

Primary leukemic erythroid cells (designated SFFV spleen) were obtained from the spleens of SFFV-P-infected BALB/c mice after one passage in vivo. After culturing the primary cells for 2 weeks in vitro, transformed erythroleukemia cell lines (designated SFFV MEL) were obtained.

Protein analysis. Cell lysates were prepared by resuspending cells in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg [each] of aprotinin and leupeptin/ml) followed by incubation on ice for 20 min. Insoluble components were removed by centrifugation, and protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). For some experiments, cells were starved for 16 h (HCD-57 and SR lines in IMDM with 1.5% FCS; MEL cells in DMEM with 1.5% FCS) and then stimulated with either Epo (50 to 100 U/ml) or IFN-α (500 U/ml) for 20 min or left unstimulated before being washed and resuspended in lysis buffer. In other experiments, cells were treated for 24 h with 70 μmol/liter of activated sodium orthovanadate (Sigma, St. Louis, Missouri) before being stimulated with Epo. Proteins were separated by electrophoresis on Tris-glycine gels (Invitrogen, Carlsbad, Calif.) and then transferred electrophoretically to nitrocellulose filters for Western blotting with anti-phospho-STAT1 (Cell Signaling Technology, Beverly, Mass.), anti-STAT1 (Santa Cruz Biotechnology, Santa Cruz, Calif.), antiphosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, New York), anti-SHP-1 (Upstate Biotechnology), or anti-PU.1 (Santa Cruz), followed by visualization using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, N.J.). To detect phosphorylated Jak2, cell extracts were subjected to immunoprecipitation as described previously (34) with Jak2 antibody (Upstate Biotechnology) and Western blotting carried out using antiphosphotyrosine antibody (4G10; Upstate Biotechnology).

Nuclear extracts. Cells were rinsed in phosphate-buffered saline, resuspended in ice-cold hypotonic buffer (20 mmol/liter HEPES [pH 7.9], 10 mmol/liter KCl, 1 mmol/liter $MgCl_2$, 10% glycerol, 0.5 mmol/liter dithiothreitol, 1× protease inhibitor cocktail containing 500 μM AEBSF, 1 mM leupeptin, 150 nM aprotinin, 1 μM E-64, 0.5 mM EDTA) (Calbiochem, La Jolla, Calif.) with 0.2% Nonidet P-40 (NP-40), and centrifuged for 5 min at $3,000 \times g$ to remove excess salt. The supernatant was discarded, and packed cells were resuspended in hypotonic buffer with 0.2% NP-40 to a final volume of three times the original packed volume of cells. The suspension was swelled on ice for 10 min and then transferred to microcentrifuge tubes and homogenized using disposable 1.5-ml pestles (Research Products International Corp., Mt. Prospect, Illinois) with 20 up and down strokes. Nuclei were collected by centrifugation at $2,000 \times g$ for 10 min. The supernatant was removed, and nuclei were resuspended in an equal volume of hypotonic buffer with 300 mM NaCl and homogenized with several strokes of the micro-Dounce homogenizer to prevent clumping. Suspensions were kept on ice for 20 min with occasional vortexing. Residual nuclei were removed by centrifugation at $14,000 \times g$ for 20 min at 4°C, and supernatants were collected as nuclear extracts.

Electrophoretic mobility shift assays. Nuclear extracts were used in an electrophoretic mobility shift assay (EMSA). Twenty micrograms of nuclear extract was incubated with a [γ-^{32}P]ATP-labeled double-stranded DNA fragment corresponding to the consensus binding site for STAT5 (SC-2565; Santa Cruz) (sense strand, 5′-AGATTTCTAGCAATTCAATCC-3′) or [α-^{32}P]CTP double-stranded oligonucleotides corresponding to the high-affinity SIE (m67) sequence (5′-GTCGACATTTCCCGTAAATC-3′) at room temperature for 30 min in the presence of binding buffer (13 mM HEPES [pH 7.9], 65 to 120 mM NaCl, 0.15 mM EDTA, 8% glycerol, and 1 mM dithiothreitol), 1 μg bovine serum albumin, 0.25× protease inhibitor cocktail, 0.05% NP-40, and 3 μg of poly(dI-dC). Samples were then subjected to electrophoresis using a 6% polyacrylamide gel containing 5% glycerol with 0.5× Tris-borate buffer. Gels were dried and exposed to Kodak XR-5 film at −80°C.

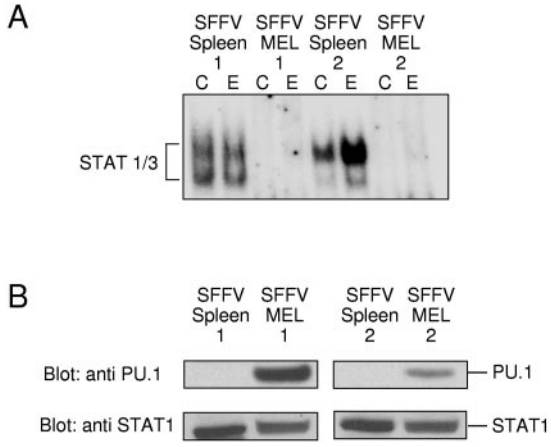

FIG. 1. Comparison of SIE DNA binding activity and PU.1 protein levels in primary leukemic splenocytes and transformed erythroleukemia cell lines from SFFV-infected mice. Primary SFFV-infected erythroleukemia cells (SFFV spleen) and transformed erythroleukemia cell lines derived from them (SFFV MEL) were prepared as described in Materials and Methods. (A) Cells were starved for 2 h and then left unstimulated (C) or stimulated with Epo (100 U/ml) (E) for 15 min. EMSA for STAT1/3 DNA binding activity was performed on nuclear extracts using an SIE probe. (B) Total cell lysates were immunoblotted with anti-PU.1 or anti-STAT1 antibody. Primary and transformed cells from two independent mice were examined.

RESULTS

Transformation of erythroid cells by SFFV is associated with a block in STAT1/3 DNA binding activity. The first stage of Friend SFFV-P-induced disease is characterized by Epo-independent proliferation and differentiation of infected erythroid precursors in the spleens of susceptible mice. Because of their ability to differentiate, the majority of these virus-infected leukemic cells are unable to grow as transformed erythroleukemia cells in vitro. However, a small percentage of these virus-infected cells express high levels of the transcription factor PU.1 due to SFFV integration, and after in vivo and in vitro selection, they can be grown as immortal erythroleukemia cell lines. These transformed cells represent the second stage of SFFV-induced disease. The erythroid hyperplasia and polycythemia occurring in the first stage of SFFV-induced disease is associated with constitutive activation of STAT proteins and their DNA binding activity (37). This is evident in Fig. 1A, where primary SFFV-infected erythroleukemia cells (designated SFFV spleen) are shown to exhibit constitutive binding to an SIE probe, which detects STATs 1 and 3, as confirmed by a supershift with specific antisera (data not shown). This is in contrast to results obtained from transformed cell lines derived from these SFFV-infected spleens (designated SFFV MEL) after 2 weeks in culture. As shown in Fig. 1A, SIE binding failed to be activated by either Epo or SFFV in the SFFV MEL

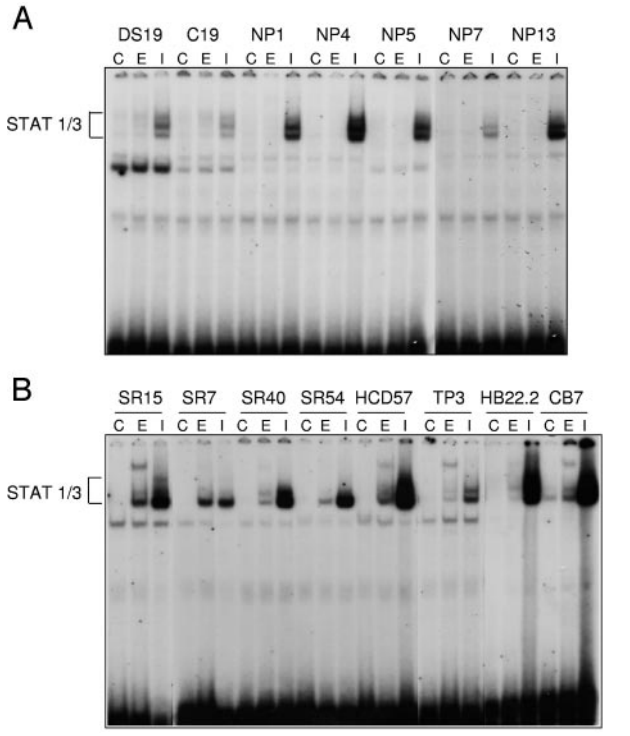

FIG. 2. Analysis of SIE DNA binding activity in erythroleukemia cell lines induced by SFFV and other retroviruses. SFFV-transformed erythroleukemia cell lines (A) and erythroleukemia cell lines derived from mice infected with F-MuLV or ME26 virus (B) were starved overnight and then left unstimulated (C) or stimulated with Epo (50 U/ml) (E) or IFN-α (500 U/ml) (I) for 15 min. EMSA for STAT1/3 DNA binding activity was performed on nuclear extracts using an SIE probe.

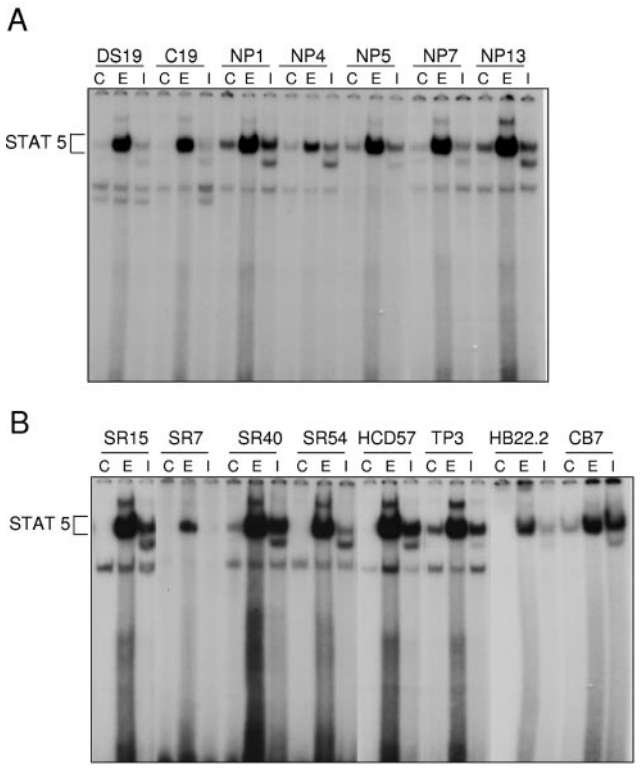

FIG. 3. Analysis of STAT5 DNA binding activity in erythroleukemia cell lines induced by SFFV and other retroviruses. SFFV-transformed erythroleukemic cells (A) and erythroleukemia cell lines derived from mice infected with F-MuLV or ME26 virus (B) were starved overnight and then left unstimulated (C) or stimulated with Epo (50 U/ml) (E) or IFN-α (500 U/ml) (I) for 15 min. EMSA for STAT5 DNA binding activity was performed on nuclear extracts using a STAT5-specific probe.

cells, although both SFFV spleens and MEL cells express equivalent levels of total STAT1 protein (Fig. 1B). The block in signal transduction pathways observed in the SFFV-transformed cells is specific for STAT1/3, since STAT5 and other Epo signal-transducing molecules, including mitogen-activated protein kinase and Akt, are still activated by both Epo and SFFV in these cells (data not shown). As previously reported, expression of the PU.1 protein was undetectable in the nontransformed SFFV-P-infected splenic erythroblasts constituting the first stage of disease but was highly expressed in transformed cell lines derived from these spleens (Fig. 1B). Thus, the block in Epo-induced differentiation that occurs in SFFV-transformed erythroleukemia cells strongly correlates with both expression of PU.1 and a block in STAT1/3 DNA binding activity.

The block in STAT1/3 DNA binding activity is specific to erythroleukemia cell lines derived from SFFV-infected mice. To confirm that a block in STAT1/3 DNA binding was a general property of erythroleukemia cells transformed by SFFV, seven independent erythroleukemia cell lines derived from SFFV-infected mice were examined. As shown in Fig. 2A, we failed to detect constitutive (lanes C) or Epo-induced (lanes E) SIE DNA binding activity in any of these lines. However, stimulation of each SFFV-transformed erythroleukemia cell line with IFN-α (lanes I) resulted in SIE DNA binding activity. To determine whether this block in STAT1/3 DNA binding activity was common among erythroleukemia cell lines or unique to those induced by SFFV, we examined erythroleukemia cells lines derived from mice infected with Friend MuLV or the *gag-myb-ets*-expressing ME26 virus. Unlike SFFV-induced erythroleukemia cell lines, those induced by Friend MuLV (F-MuLV) or ME26 virus, which do not express PU.1 (data not shown), fail to show a block in SIE DNA binding activity in response to Epo. As shown in Fig. 2B, erythroleukemia cell lines derived from F-MuLV-infected mice (HCD-57, TP3, HB22.2, and CB7 cells) or mice infected with ME26 virus (SR7, SR15, SR40, and SR54) showed SIE DNA binding after stimulation with either Epo (lanes E) or IFN-α (lanes I). These results indicate that the block in Epo-induced SIE DNA binding activity is unique to SFFV-transformed erythroleukemia cells and that SFFV-transformed cells are capable of activating SIE DNA binding activity in response to other inducers.

Erythroleukemia cell lines from SFFV-infected mice show constitutive STAT5 DNA binding activity. Because we did not detect STAT1/3 DNA binding in SFFV-transformed erythroleukemia cells either in the absence or presence of Epo, STAT5 activation was also examined. As shown in Fig. 3A, all of the SFFV-transformed erythroleukemia cell lines examined

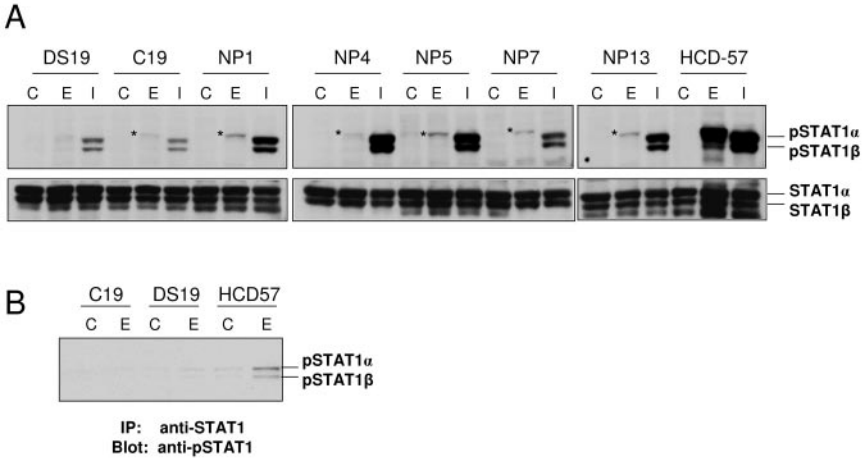

FIG. 4. Analysis of tyrosine phosphorylation of STAT1 in SFFV-transformed erythroleukemia cell lines. (A) SFFV-transformed erythroleukemia cells were left unstimulated (C) or stimulated with Epo (100 U/ml) (E) or IFN-α (500 U/ml) (I) for 15 min. Total cell lysates were then immunoblotted with either anti-phospho-STAT1 (tyrosine 701) (upper panel) or anti-STAT1 antibody (lower panel). Asterisks indicate nonspecific bands. HCD-57 cells (far right), derived from an F-MuLV-infected mouse, were used as a positive control. (B) To demonstrate that the nonspecific band marked by an asterisk in panel A was not STAT1, SFFV-transformed C19 and DS19 cells were left unstimulated (C) or stimulated with Epo (100 U/ml) (E) for 15 min, and then total cell lysates were immunoprecipitated with anti-STAT1 antibody. The immunoprecipitated proteins were then separated by electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-phospho-STAT1 antibody. HCD-57 cells were used as a positive control.

showed activation of STAT5 DNA binding activity in response to Epo (lanes E) and most showed constitutive activation of STAT5 (lanes C). STAT5 DNA binding activity was also induced in all F-MuLV- and ME26-transformed erythroleukemia cells after stimulation with Epo (Fig. 3B, lanes E). Interestingly, stimulation with IFN-α strongly induced STAT 5 DNA binding activity in many of the erythroleukemia cell lines examined (Fig. 3B, lanes I). These results indicate that induction of STAT5 DNA binding activity by SFFV or Epo is not impaired in SFFV-transformed erythroleukemia cells.

STAT1 α and β are not tyrosine phosphorylated in SFFV-transformed erythroleukemia cell lines before or after stimulation with Epo. STAT proteins are phosphorylated in the cytoplasm and then transported to the nucleus where they bind DNA. To test whether the block in STAT1 activation in SFFV-transformed erythroleukemia cells occurs at the level of DNA binding or phosphorylation of the protein, we performed Western blot analysis using anti-phospho-STAT1 antibody, which detects phosphorylated tyrosine 701 of the α and β isoforms of STAT1. As shown in Fig. 4A, STAT1 α and β are strongly tyrosine phosphorylated in the F-MuLV-transformed cell line HCD-57 (far right) after stimulation with Epo (lane E) as well as IFN-α (lane I). In contrast, we failed to detect either constitutive (lanes C) or Epo-induced (lanes E) STAT1 α and β tyrosine phosphorylation in SFFV-transformed erythroleukemia cell lines (Fig. 4A), although IFN-α stimulation (lanes I) induced tyrosine phosphorylation of STAT1 α and β in these lines. One band in the Epo-stimulated cells in Fig. 4A is a nonspecific band, as indicated by its failure to be precipitated by anti-STAT1 antibody (Fig. 4B). Unlike STAT1, STAT5 was constitutively tyrosine phosphorylated in all of the SFFV-transformed erythroleukemia cell lines examined (data not shown). Because of the difficulty in detecting tyrosine-phosphorylated STAT3 even in cells that show high SIE DNA binding activity, such as HCD-57 cells, we were unable to draw any conclusions about the phosphorylation status of STAT3 in SFFV-transformed erythroleukemia cells.

Jak2 is constitutively activated in SFFV-transformed erythroleukemic cell lines. Jak2 is the major regulator of STAT protein tyrosine phosphorylation in erythroid cells (58). Although STAT5 is phosphorylated in SFFV-transformed erythroid cells, its phosphorylation is not dependent upon Epo and may be Jak2 independent. To determine whether the block in STAT1 phosphorylation in SFFV-transformed cells was due

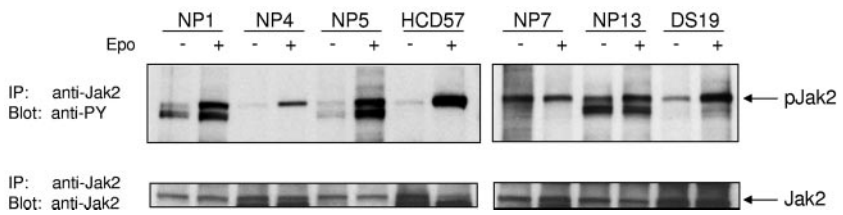

FIG. 5. Analysis of Jak2 phosphorylation in SFFV-transformed erythroleukemia cell lines. SFFV transformed erythroleukemia cells were left unstimulated (−) or stimulated with Epo (100 U/ml) (+) for 15 min, and total cell lysates were immunoprecipitated with anti-Jak2 antibody. The immunoprecipitated (IP) proteins were then separated electrophoretically, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine (anti-PY) antibody or anti-Jak2 antibody. HCD-57 cells were used as a positive control.

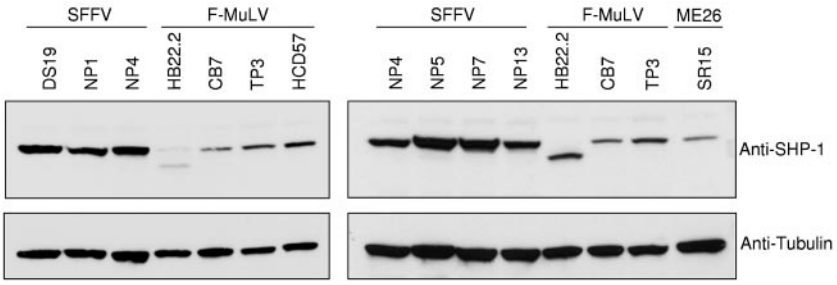

FIG. 6. Comparison of SHP-1 expression in erythroleukemia cell lines from mice infected with SFFV and other retroviruses. Total cell lysates prepared from erythroleukemia cell lines from SFFV-infected, F-MuLV-infected, or ME26 virus-infected mice were immunoblotted with anti-SHP-1 antibody. The membranes were stripped and then reimmunoblotted with antitubulin antibody.

to a block in Jak2 activation, we tested these cells for phosphorylation of Jak2 in response to Epo. Cell lysates from SFFV-transformed cells were immunoprecipitated with anti-Jak2 antibody, and Western blot analysis was carried out using antiphosphotyrosine antibody. As shown in Fig. 5, Jak2 tyrosine phosphorylation could be detected after Epo stimulation in all of the SFFV-transformed erythroleukemia cell lines examined and was actually constitutive in many of the lines. However, the level of tyrosine-phosphorylated Jak2 in SFFV-transformed erythroleukemia cells was generally lower than that detected in the F-MuLV-transformed erythroleukemia cell line HCD-57, which shows high levels of STAT1 phosphorylation upon Epo stimulation (Fig. 4). These results indicate that the failure of STAT1 to be phosphorylated in Epo-stimulated SFFV-transformed erythroid cells is not due to the failure of Epo to activate Jak2 in these cells.

SFFV-transformed erythroleukemia cell lines show high expression of SHP-1. To determine whether the failure of STAT1 to be phosphorylated in SFFV-transformed erythroleukemia cells was due to activation of a negative regulator of Jak/STAT signaling, we examined the cells for expression of the tyrosine phosphatase SHP-1, which has previously been shown to negatively regulate erythroid signal transduction pathways (21, 51, 60). Using Western blot analysis with an SHP-1 antibody, we observed that SHP-1 is highly expressed in all SFFV-transformed erythroleukemia cell lines (Fig. 6). On the other hand, F-MuLV- or ME26-transformed erythroleukemia cell lines (Fig. 6) and primary erythroleukemic spleens from SFFV-infected mice (data not shown) exhibited relatively lower levels of expression of SHP-1. Treatment with Epo or IFN-α did not alter the level of expression of SHP-1 in SFFV-transformed cells (data not shown). When we treated the SFFV-transformed erythroleukemia cell lines NP4 and NP5 with the tyrosine phosphatase inhibitor sodium orthovanadate (70 μmol/liter) for 48 h (Fig. 7), tyrosine phosphorylation of STAT1α and β was strongly induced either in the presence or absence of Epo. However, treatment with sodium orthovanadate was unable to restore STAT1/3 DNA binding activity in these cells (data not shown), perhaps because PU.1 levels remained high. These results suggest that the failure to detect tyrosine-phosphorylated STAT1 in SFFV-transformed erythroleukemia cells may be due to dephosphorylation of STAT1 by a tyrosine phosphatase such as SHP-1.

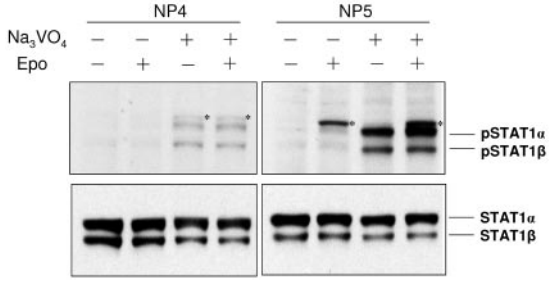

FIG. 7. Tyrosine phosphorylation of STAT1 is induced in SFFV-transformed erythroleukemia cell lines treated with a tyrosine phosphatase inhibitor. The SFFV-transformed erythroleukemia cell lines NP4 and NP5 cells were treated with 70 μmol/liter sodium orthovanadate (Na_3VO_4) for 24 h (+) or left untreated (−). Cells were left unstimulated (−) or stimulated with Epo (50 U/ml) for 15 min (+). Cell lysates were immunoblotted with either anti-phospho-STAT1 or anti-STAT1 antibody. Asterisks represent nonspecific bands (see legend to Fig. 4).

DISCUSSION

Binding of Epo to the Epo receptor results in the tyrosine phosphorylation of STATs 1, 3, and 5 (15, 37, 41, 57), which then form dimers, move to the nucleus, and bind specific DNA sequences. We previously showed that infection of the Epo-dependent erythroleukemia cell line HCD-57 with SFFV resulted in constitutive activation of these STATs (36, 37), and in this study, we show that primary virus-infected leukemic splenocytes from the first stage of SFFV-induced erythroleukemia also exhibit constitutive activation of STATs 1, 3, and 5. However, when these primary SFFV-infected leukemic splenocytes are transplanted to syngeneic mice and grown in vitro to select for the second stage of erythroleukemia, the resulting transformed erythroleukemia cell lines fail to show STAT1/3 DNA binding, even after stimulation with Epo (Fig. 1 and 2). Although none of the SFFV-transformed erythroleukemia cell lines that we examined exhibited STAT1/3 activation in response to Epo, they all showed Epo activation of STAT5, with all but one of the lines showing constitutive activation of STAT5. Furthermore, we could detect constitutive activation of mitogen-activated protein kinase and Akt in these cells (data not shown). Thus, a failure to activate STAT1/3 in SFFV-transformed erythroleukemia cells does not appear to be the result of defective Epo signaling from the EpoR in these cells. A block in the activation of STAT1/3 DNA-binding is not a general property of erythroleukemia cell lines, since those de-

rived from mice infected with Friend MuLV or ME26 virus did not exhibit this block. Compared with erythroleukemia cell lines derived from mice infected with F-MuLV and ME26 virus, SFFV-transformed erythroleukemia cells represent a later stage of hematopoietic cell differentiation (38, 46, 52) and, unlike the other erythroleukemia cell lines, express high levels of the Ets-related transcription factor PU.1 (50).

Our data indicate that the defect in Epo activation of STAT 1/3 DNA binding activity in SFFV-transformed erythroleukemia cells is specifically at the level of tyrosine phosphorylation, since STAT1 was not tyrosine phosphorylated in response to either SFFV or Epo stimulation in these cells. In contrast, IFN-α stimulation of SFFV-transformed erythroleukemia cells induced STAT1 tyrosine phosphorylation, suggesting that the STAT1 proteins in these cells are not dysfunctional. Rather, the SFFV transformed erythroleukemia cells appear to express inhibitors that interfere with Epo-induced STAT1 tyrosine phosphorylation. A number of inhibitors of STAT signaling have previously been identified. These include SOCS (suppressors of cytokine signaling) (17, 24, 27, 32, 55, 61), SS1 (STAT-induced STAT inhibitors) (27, 32), and JAB (Jak binding) (11, 49) proteins, which indirectly exert negative regulation on STAT proteins by specifically binding to and inhibiting the tyrosine kinase activity of cytoplasmic Jaks, preventing STAT phosphorylation. Since we could detect constitutive or Epo-induced Jak2 phosphorylation in all of the SFFV-transformed erythroleukemia cells, SOCS/SS1/JAB proteins do not appear to be responsible for the failure of STAT1 to be phosphorylated in SFFV-transformed erythroid cells. Also, the PIAS (protein inhibitor of activated STATs) family of STAT inhibitors, which inhibit STAT signaling by binding to phosphorylated dimers of STAT proteins (6, 23), would not be responsible for the failure of STAT1 signaling in SFFV-transformed erythroleukemia cells, since we failed to detect phosphorylated STAT1 in these cells. Thus, known inhibitors of STAT signaling do not appear to be involved in blocking STAT1 and 3 from binding DNA in SFFV-transformed erythroleukemia cells.

Based on these observations, we looked at expression of other negative regulators of EpoR signaling. One such protein is the hematopoietic tyrosine phosphatase SHP-1. SHP-1 is predominantly expressed in hematopoietic cells and epithelial cells (42) and is known to be recruited to a number of cytokine receptors, including the EpoR and the IFN-α/β receptor (8, 21, 60). Although SHP-1 has been shown to inhibit EpoR signaling associated with cell proliferation (21, 60), it also plays a significant role in erythroid cell differentiation, since its overexpression is associated with inhibition of erythroid cell differentiation and its inhibition with enhanced hemoglobinization (2, 51). Furthermore, motheaten mice, which contain a loss-of-function mutation in SHP-1, show high numbers of the late erythroid precursor CFU-E (53), and members of a Finnish family who have a mutation in the EpoR gene that prevents binding of SHP-1 express elevated levels of mature red blood cells (10). When we compared various mouse erythroleukemia cell lines for expression of SHP-1, those from SFFV-infected mice showed much higher levels than those from F-MuLV- and ME26 virus-infected mice, and treatment of the SFFV-transformed erythroleukemia cell lines with sodium orthovanadate, a specific tyrosine phosphatase inhibitor, restored STAT1 tyrosine phosphorylation. These data suggest that SHP-1 may be interfering with the activation of STAT1/3 in SFFV-transformed erythroleukemia cells. Consistent with this idea are studies using SHP-1-deficient mice, which showed that STAT1 phosphorylation increases in cells from these mice in response to IFN-α/β (8). Also, our recent studies using the SFFV MEL cell line DS19 showed that when PU.1 and SHP-1 levels are reduced in conjunction with chemically induced differentiation of these cells, Epo-dependent STAT1/3 DNA binding activity is restored (data not shown). Previous studies have shown that SHP-1 can dephosphorylate Jak2 (18), which in turn could block STAT phosphorylation. However, in SFFV-transformed erythroleukemia cells, SHP-1 does not appear to be dephosphorylating Jak2 because we detected high levels of tyrosine-phosphorylated Jak2 in these cells. Thus, SHP-1 may be directly dephosphorylating STAT1 in the SFFV-transformed erythroleuekemia cells. This idea is supported by a study showing that, in liver cells stimulated with growth hormone, SHP-1 binds to and may be directly dephosphorylating tyrosine-phosphorylated STAT5b (44). A recent study using STAT1-deficient mice demonstrated that STAT1 plays an important role in the regulation of erythropoiesis (16). Thus, dephosphorylation of STAT1 in SFFV-transformed erythroleukemia cell lines cells due to high SHP-1 expression may contribute to their block in erythroid cell differentiation. Although high expression of SHP-1 is associated with the lack of Epo-induced STAT1 tyrosine phosphorylation in SFFV MEL cells, it does not alter STAT1 tyrosine phosphorylation induced in these cells by IFN-α, suggesting that Epo- and IFN-α-induced modifications of STAT1 proteins are different, resulting in differences in sensitivity to SHP-1. Overexpression of SHP-1 has been shown to occur in many types of cancer (28, 56, 59), and this may contribute to the transformed phenotype by blocking differentiation.

Unlike normal erythroid cells, SFFV-transformed erythroleukemia cells express high levels of the transcription factor PU.1, and among the erythroleukemia cells that we examined in this study, we observed a direct correlation between the expression of PU.1, the high expression of SHP-1, and a block in STAT1/3 DNA binding activity. A recent paper showed that SHP-1 expression is significantly reduced in PU.1$^{-/-}$ erythroid cells using microarray analysis (13), suggesting that SHP-1 may be regulated by the PU.1 transcription factor. A number of reports (33, 45, 63, 64) have shown that PU.1, when it is overexpressed in erythroleukemia cell lines, blocks erythroid terminal differentiation by physically interacting with and blocking the action of the erythroid transcription factor GATA-1, which positively regulates numerous erythroid-restricted genes. Our results suggest that PU.1's mechanism of action may also extend to activation of SHP-1 and inhibition of STAT 1/3 DNA binding activity. Inhibition of cyclin-dependent kinases by activation of CDK inhibitors appears to be essential for the induction of cell cycle arrest and the initiation of terminal erythroid differentiation (25, 26), and roles for STAT proteins in negatively regulating the cell cycle have been demonstrated. For example, STAT3 induced by granulocyte colony-stimulating factor has been shown to transactivate the cyclin-dependent kinase inhibitor p27^{KIP-1} through a putative STAT-binding site in its promoter (9), and activation of STAT1 in response to IFN-γ correlates with upregulation of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ (4, 5).

Thus, inhibition of STAT1/3 DNA binding in SFFV-transformed erythroleukemia cells may prevent the upregulation of cyclin-dependent kinase inhibitors that are required for cell cycle arrest and the initiation of terminal differentiation.

This study suggests a potential new mechanism by which inappropriate expression of the PU.1 transcription factor in erythroid cells can alter specific signal transducing molecules, STATs 1 and 3, to block terminal differentiation and favor the outgrowth of transformed cells. Mechanisms to restore STAT1/3 DNA binding in these cells may, therefore, favor differentiation and the extinction of the transformed phenotype.

ACKNOWLEDGMENTS

We thank Yaacov Ben-David for his generous gift of the CB7 and HB22.2 cell lines and Joan Cmarik for critical reading of the manuscript.

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

REFERENCES

1. **Ben-David, Y., and A. Bernstein.** 1991. Friend virus-induced erythroleukemia and the multistage nature of cancer. Cell **66:**831–834.
2. **Bittorf, T., J. Seiler, Z. Zhang, R. Jaster, and J. Brock.** 1999. SHP1 protein tyrosine phosphatase negatively modulates erythroid differentiation and suppression of apoptosis in J2E erythroleukemic cells. Biol. Chem. **380:**1201–1209.
3. **Casadevall, N., C. Lacombe, O. Muller, S. Gisselbrecht, and P. Mayeux.** 1991. Multimeric structure of the membrane erythropoietin receptor of murine erythroleukemia cells (Friend cells). Cross-linking of erythropoietin with the spleen focus-forming virus envelope protein. J. Biol. Chem. **266:**16015–16020.
4. **Chen, B., L. He, V. H. Savell, J. J. Jenkins, and D. M. Parham.** 2000. Inhibition of the interferon-gamma/signal transducers and activators of transcription (STAT) pathway by hypermethylation at a STAT-binding site in the p21WAF1 promoter region. Cancer Res. **60:**3290–3298.
5. **Chin, Y. E., M. Kitagawa, W. C. Su, Z. H. You, Y. Iwamoto, and X. Y. Fu.** 1996. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science **272:**719–722.
6. **Chung, C. D., J. Liao, B. Liu, X. Rao, P. Jay, P. Berta, and K. Shuai.** 1997. Specific inhibition of Stat3 signal transduction by PIAS3. Science **278:**1803–1805.
7. **Damen, J. E., H. Wakao, A. Miyajima, J. Krosl, R. K. Humphries, R. L. Cutler, and G. Krystal.** 1995. Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. EMBO J. **14:**5557–5568.
8. **David, M., H. E. Chen, S. Goelz, A. C. Larner, and B. G. Neel.** 1995. Differential regulation of the alpha/beta interferon-stimulated Jak/Stat pathway by the SH2 domain-containing tyrosine phosphatase SHPTP1. Mol. Cell. Biol. **15:**7050–7058.
9. **de Koning, J. P., A. A. Soede-Bobok, A. C. Ward, A. M. Schelen, C. Antonissen, D. van Leeuwen, B. Lowenberg, and I. P. Touw.** 2000. STAT3-mediated differentiation and survival of myeloid cells in response to granulocyte colony-stimulating factor: role for the cyclin-dependent kinase inhibitor p27(Kip1). Oncogene **19:**3290–3298.
10. **de la Chapelle, A., A. L. Traskelin, and E. Juvonen.** 1993. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. Proc. Natl. Acad. Sci. USA **90:**4495–4499.
11. **Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, and A. Yoshimura.** 1997. A new protein containing an SH2 domain that inhibits JAK kinases. Nature **387:**921–924.
12. **Ferro, F. E., Jr., S. L. Kozak, M. E. Hoatlin, and D. Kabat.** 1993. Cell surface site for mitogenic interaction of erythropoietin receptors with the membrane glycoprotein encoded by Friend erythroleukemia virus. J. Biol. Chem. **268:**5741–5747.
13. **Fisher, R. C., W. B. Slayton, C. Chien, S. M. Guthrie, C. Bray, and E. W. Scott.** 2004. PU.1 supports proliferation of immature erythroid progenitors. Leuk. Res. **28:**83–89.
14. **Gobert, S., S. Chretien, F. Gouilleux, O. Muller, C. Pallard, I. Dusanter-Fourt, B. Groner, C. Lacombe, S. Gisselbrecht, and P. Mayeux.** 1996. Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation. EMBO J. **15:**2434–2441.
15. **Gouilleux, F., C. Pallard, I. Dusanter-Fourt, H. Wakao, L. A. Haldosen, G. Norstedt, D. Levy, and B. Groner.** 1995. Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. EMBO J. **14:**2005–2013.
16. **Halupa, A., M. L. Bailey, K. Huang, N. N. Iscove, D. E. Levy, and D. L. Barber.** 2005. A novel role for STAT1 in regulating murine erythropoiesis: deletion of STAT1 results in overall reduction of erythroid progenitors and alters their distribution. Blood **105:**552–561.
17. **Hilton, D. J., R. T. Richardson, W. S. Alexander, E. M. Viney, T. A. Willson, N. S. Sprigg, R. Starr, S. E. Nicholson, D. Metcalf, and N. A. Nicola.** 1998. Twenty proteins containing a C-terminal SOCS box form five structural classes. Proc. Natl. Acad. Sci. USA **95:**114–119.
18. **Jiao, H., K. Berrada, W. Yang, M. Tabrizi, L. C. Platanias, and T. Yi.** 1996. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. Mol. Cell. Biol. **16:**6985–6992.
19. **Kirito, K., M. Uchida, M. Yamada, Y. Miura, and N. Komatsu.** 1997. A distinct function of STAT proteins in erythropoietin signal transduction. J. Biol. Chem. **272:**16507–16513.
20. **Klingmuller, U., S. Bergelson, J. G. Hsiao, and H. F. Lodish.** 1996. Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. Proc. Natl. Acad. Sci. USA **93:**8324–8328.
21. **Klingmuller, U., U. Lorenz, L. C. Cantley, B. G. Neel, and H. F. Lodish.** 1995. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. Cell **80:**729–738.
22. **Li, J. P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore.** 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature **343:**762–764.
23. **Liu, B., J. Liao, X. Rao, S. A. Kushner, C. D. Chung, D. D. Chang, and K. Shuai.** 1998. Inhibition of Stat1-mediated gene activation by PIAS1. Proc. Natl. Acad. Sci. USA **95:**10626–10631.
24. **Matsumoto, A., M. Masuhara, K. Mitsui, M. Yokouchi, M. Ohtsubo, H. Misawa, A. Miyajima, and A. Yoshimura.** 1997. CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. Blood **89:**3148–3154.
25. **Matushansky, I., F. Radparvar, and A. I. Skoultchi.** 2000. Manipulating the onset of cell cycle withdrawal in differentiated erythroid cells with cyclin-dependent kinases and inhibitors. Blood **96:**2755–2764.
26. **Matushansky, I., F. Radparvar, and A. I. Skoultchi.** 2000. Reprogramming leukemic cells to terminal differentiation by inhibiting specific cyclin-dependent kinases in G1. Proc. Natl. Acad. Sci. USA **97:**14317–14322.
27. **Minamoto, S., K. Ikegame, K. Ueno, M. Narazaki, T. Naka, H. Yamamoto, T. Matsumoto, H. Saito, S. Hosoe, and T. Kishimoto.** 1997. Cloning and functional analysis of new members of STAT induced STAT inhibitor (SSI) family: SSI-2 and SSI-3. Biochem. Biophys. Res. Commun. **237:**79–83.
28. **Mok, S. C., T. T. Kwok, R. S. Berkowitz, A. J. Barrett, and F. W. Tsui.** 1995. Overexpression of the protein tyrosine phosphatase, nonreceptor type 6 (PTPN6), in human epithelial ovarian cancer. Gynecol. Oncol. **57:**299–303.
29. **Moreau-Gachelin, F., A. Tavitian, and P. Tambourin.** 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. Nature **331:**277–280.
30. **Muszynski, K. W., T. Ohashi, C. Hanson, and S. K. Ruscetti.** 1998. Both the polycythemia- and anemia-inducing strains of Friend spleen focus-forming virus induce constitutive activation of the Raf-1/mitogen-activated protein kinase signal transduction pathway. J. Virol. **72:**919–925.
31. **Muszynski, K. W., D. Thompson, C. Hanson, R. Lyons, A. Spadaccini, and S. K. Ruscetti.** 2000. Growth factor-independent proliferation of erythroid cells infected with Friend spleen focus-forming virus is protein kinase C dependent but does not require Ras-GTP. J. Virol. **74:**8444–8451.
32. **Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, and T. Kishimoto.** 1997. Structure and function of a new STAT-induced STAT inhibitor. Nature **387:**924–929.
33. **Nerlov, C., E. Querfurth, H. Kulessa, and T. Graf.** 2000. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. Blood **95:**2543–2551.
34. **Nishigaki, K., C. Hanson, T. Ohashi, D. Thompson, K. Muszynski, and S. Ruscetti.** 2000. Erythroid cells rendered erythropoietin independent by infection with Friend spleen focus-forming virus show constitutive activation of phosphatidylinositol 3-kinase and Akt kinase: involvement of insulin receptor substrate-related adapter proteins. J. Virol. **74:**3037–3045.
35. **Nishigaki, K., D. Thompson, C. Hanson, T. Yugawa, and S. Ruscetti.** 2001. The envelope glycoprotein of Friend spleen focus-forming virus covalently interacts with and constitutively activates a truncated form of the receptor tyrosine kinase Stk. J. Virol. **75:**7893–7903.
36. **Ohashi, T., M. Masuda, and S. K. Ruscetti.** 1997. Constitutive activation of Stat-related DNA-binding proteins in erythroid cells by the Friend spleen focus-forming virus. Leukemia **11**(Suppl. 3)**:**251–254.
37. **Ohashi, T., M. Masuda, and S. K. Ruscetti.** 1995. Induction of sequence-specific DNA-binding factors by erythropoietin and the spleen focus-forming virus. Blood **85:**1454–1462.
38. **Oliff, A., S. Ruscetti, E. C. Douglass, and E. Scolnick.** 1981. Isolation of

transplantable erythroleukemia cells from mice infected with helper-independent Friend murine leukemia virus. Blood **58:**244–254.
39. **Paul, R., S. Schuetze, S. L. Kozak, and D. Kabat.** 1989. A common site for immortalizing proviral integrations in Friend erythroleukemia: molecular cloning and characterization. J. Virol. **63:**4958–4961.
40. **Paul, R., S. Schuetze, S. L. Kozak, C. A. Kozak, and D. Kabat.** 1991. The Sfpi-1 proviral integration site of Friend erythroleukemia encodes the ets-related transcription factor Pu.1. J. Virol. **65:**464–467.
41. **Penta, K., and S. T. Sawyer.** 1995. Erythropoietin induces the tyrosine phosphorylation, nuclear translocation, and DNA binding of STAT1 and STAT5 in erythroid cells. J. Biol. Chem. **270:**31282–31287.
42. **Plutzky, J., B. G. Neel, and R. D. Rosenberg.** 1992. Isolation of a src homology 2-containing tyrosine phosphatase. Proc. Natl. Acad. Sci. USA **89:**1123–1127.
43. **Quelle, F. W., D. Wang, T. Nosaka, W. E. Thierfelder, D. Stravopodis, Y. Weinstein, and J. N. Ihle.** 1996. Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. Mol. Cell. Biol. **16:**1622–1631.
44. **Ram, P. A., and D. J. Waxman.** 1997. Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. J. Biol. Chem. **272:**17694–17702.
45. **Rekhtman, N., F. Radparvar, T. Evans, and A. I. Skoultchi.** 1999. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. Genes Dev. **13:**1398–1411.
46. **Ruscetti, S., R. Aurigemma, C. C. Yuan, S. Sawyer, and D. G. Blair.** 1992. Induction of erythropoietin responsiveness in murine hematopoietic cells by the gag-myb-ets-containing ME26 virus. J. Virol. **66:**20–26.
47. **Ruscetti, S. K.** 1999. Deregulation of erythropoiesis by the Friend spleen focus-forming virus. Int. J. Biochem. Cell Biol. **31:**1089–1109.
48. **Ruscetti, S. K., N. J. Janesch, A. Chakraborti, S. T. Sawyer, and W. D. Hankins.** 1990. Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. J. Virol. **64:**1057–1062.
49. **Sakamoto, H., H. Yasukawa, M. Masuhara, S. Tanimura, A. Sasaki, K. Yuge, M. Ohtsubo, A. Ohtsuka, T. Fujita, T. Ohta, Y. Furukawa, S. Iwase, H. Yamada, and A. Yoshimura.** 1998. A Janus kinase inhibitor, JAB, is an interferon-gamma-inducible gene and confers resistance to interferons. Blood **92:**1668–1676.
50. **Schuetze, S., R. Paul, B. C. Gliniak, and D. Kabat.** 1992. Role of the PU.1 transcription factor in controlling differentiation of Friend erythroleukemia cells. Mol. Cell. Biol. **12:**2967–2975.
51. **Sharlow, E. R., R. Pacifici, J. Crouse, J. Batac, K. Todokoro, and D. M. Wojchowski.** 1997. Hematopoietic cell phosphatase negatively regulates erythropoietin-induced hemoglobinization in erythroleukemic SKT6 cells. Blood **90:**2175–2187.
52. **Shibuya, T., and T. W. Mak.** 1983. Isolation and induction of erythroleukemic cell lines with properties of erythroid progenitor burst-forming cell (BFU-E) and erythroid precursor cell (CFU-E). Proc. Natl. Acad. Sci. USA **80:**3721–3725.
53. **Shultz, L. D., P. A. Schweitzer, T. V. Rajan, T. Yi, J. N. Ihle, R. J. Matthews, M. L. Thomas, and D. R. Beier.** 1993. Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. Cell **73:**1445–1454.
54. **Singer, D., M. Cooper, G. M. Maniatis, P. A. Marks, and R. A. Rifkind.** 1974. Erythropoietic differentiation in colonies of cells transformed by Friend virus. Proc. Natl. Acad. Sci. USA **71:**2668–2670.
55. **Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton.** 1997. A family of cytokine-inducible inhibitors of signalling. Nature **387:**917–921.
56. **Tsui, H. W., K. Hasselblatt, A. Martin, S. C. Mok, and F. W. Tsui.** 2002. Molecular mechanisms underlying SHP-1 gene expression. Eur. J. Biochem. **269:**3057–3064.
57. **Wakao, H., N. Harada, T. Kitamura, A. L. Mui, and A. Miyajima.** 1995. Interleukin 2 and erythropoietin activate STAT5/MGF via distinct pathways. EMBO J. **14:**2527–2535.
58. **Witthuhn, B. A., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle.** 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell **74:**227–236.
59. **Wu, C., M. Sun, L. Liu, and G. W. Zhou.** 2003. The function of the protein tyrosine phosphatase SHP-1 in cancer. Gene **306:**1–12.
60. **Yi, T., J. Zhang, O. Miura, and J. N. Ihle.** 1995. Hematopoietic cell phosphatase associates with erythropoietin (Epo) receptor after Epo-induced receptor tyrosine phosphorylation: identification of potential binding sites. Blood **85:**87–95.
61. **Yoshimura, A., T. Ohkubo, T. Kiguchi, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Hara, and A. Miyajima.** 1995. A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. EMBO J. **14:**2816–2826.
62. **Yuan, C. C., N. Kan, K. J. Dunn, T. S. Papas, and D. G. Blair.** 1989. Properties of a murine retroviral recombinant of avian acute leukemia virus E26: a murine fibroblast assay for v-ets function. J. Virol. **63:**205–215.
63. **Zhang, P., G. Behre, J. Pan, A. Iwama, N. Wara-Aswapati, H. S. Radomska, P. E. Auron, D. G. Tenen, and Z. Sun.** 1999. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. Proc. Natl. Acad. Sci. USA **96:**8705–8710.
64. **Zhang, P., X. Zhang, A. Iwama, C. Yu, K. A. Smith, B. U. Mueller, S. Narravula, B. E. Torbett, S. H. Orkin, and D. G. Tenen.** 2000. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. Blood **96:**2641–2648.