Kit-activating mutations cooperate with Spi-1/PU.1 overexpression to promote tumorigenic progression during erythroleukemia in mice

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Summary

The erythroleukemia developed by *spi-1/PU.1* transgenic mice is a multistage process characterized by an early arrest of the proerythroblast differentiation followed later on by malignant transformation. Herein, we report the presence of acquired mutations in the *SCF* receptor gene (*Kit*) in 86% of tumors isolated during the late stage of the disease. Kit mutations affect codon 814 or 818. Ectopic expression of Kit mutants in nonmalignant proerythroblasts confers erythropoietin independence and tumorigenicity to cells. Using PP1, PP2, and imatinib mesylate, we show that Kit mutants are responsible for the autonomous expansion of malignant cells via Erk1/2 and PI3K/Akt activations. These findings represent a proof of principle for oncogenic cooperativity between one proliferative and one differentiation blocking event for the development of an overt leukemia.

Introduction

Human tumors, as well as murine cancer models, progress by the accumulation of genetic abnormalities in somatic cells, allowing them to escape from control mechanisms involved in cell differentiation, growth, and death. The acute Friend erythroleukemia that is induced in mice by the Friend virus spleen focus-forming virus (SFFV) has provided an important model to understand the multistage nature of leukemogenesis. The Friend disease evolves in two stages. The first stage is characterized by a growth factor-independent polyclonal proliferation of erythroid progenitors, most of them undergoing terminal erythroid differentiation. The late stage is marked by the emergence of leukemic proerythroblastic clones, which are unable to differentiate into mature red blood cells and are tumorigenic in vivo (Ben-David and Bernstein, 1991). Three molecular events underlying the progression of the Friend leukemia have been identified. During the early phase of the disease, the activation of the erythropoietin (Epo) receptor (EpoR) by the gp55 glycoprotein encoded by the SFFV *env* gene makes the growth and differentiation of the proerythroblast Epo independent (Aizawa et al., 1990; Li et al., 1990). In the late phase of the disease, the transcriptional activation of the *spi-1* gene by SFFV insertional mutagenesis is correlated with the emergence of malignant proerythroblastic clones (Moreau-Gachelin et al., 1988, 1989; Paul et al., 1989). In addition, mutations in the *p53* gene resulting in a loss of function are also detected in highly tumorigenic proerythroblasts isolated late in disease pathogenesis (Ben-David et al., 1988; Munroe et al., 1990).

The product of the *spi-1* gene is the transcription factor PU.1, a member of the ETS protein family (Goebl et al., 1990; Klemsz et al., 1990). *PU.1* deletion causes failure in lymphoid and myeloid development, and extinction of PU.1 is required for normal erythroid development (McKercher et al., 1996; Nutt et al., 2005). Moreover, according to the cell lineage, the function of PU.1 is dose dependent (DeKoter and Singh, 2000). These spatial and temporal differences for Spi-1/PU.1 function may provide the basis for understanding the opposing roles of PU.1 in leukemogenesis. Hypomorphic mutations of PU.1 have been identified in rare human acute myeloid leukemia (AML), as well

SIGNIFICANCE

The "two-hit" model for leukemogenesis predicts that acute myeloid leukemia (AML) is the consequence of a collaboration between two classes of mutations, one conferring a proliferative advantage and one blocking differentiation. The pathogenesis of acute erythroleukemia in *spi-1/PU.1* transgenic mice brings a direct validation to this concept. Spi-1/PU.1 overexpression in vivo induces a primary impairment of erythroblast differentiation and creates conditions to select cooperating oncogenic events. The finding of activating mutations in *kit*, which are detected in 86% of tumors, provides direct evidence for a second hit that induces proliferative signals that contribute to malignancy. This model recapitulates many characteristics of AML in humans and provides tools to elucidate secondary effects of these mutations during disease initiation and progression.

as in radiation-induced myeloid leukemia in mice (Cook et al., 2004; Mueller et al., 2002; Suraweera et al., 2005). The targeted knockdown of PU.1 demonstrates that a residual expression of PU.1 in mice also induces AML (Rosenbauer et al., 2004). In the murine Friend erythroleukemia, the upregulation of Spi-1/PU.1 is oncogenic (Moreau-Gachelin et al., 1988). The consequences of a Spi-1 overexpression in proerythroblasts were deduced from the pathological features occurring in *spi-1* transgenic (*spi-1*-TG) mice (Moreau-Gachelin et al., 1996). *Spi-1*-TG mice develop a severe anemia and a large hepatosplenomegaly (HS1 stage) due to the proliferation of proerythroblasts blocked in their differentiation and dependent upon Epo for their survival and growth. At the onset of the disease, erythroleukemic *spi-1*-TG mice may be treated by repeated transfusions of red blood cells. However, all animals relapse with hepatosplenomegaly (HS2 stage) due to the proliferation of proerythroblasts that are blocked in their differentiation but that have acquired the ability to grow without Epo and to induce tumors in immunodeficient mice. The multistep erythroleukemic process in *spi-1*-TG mice shows that a high level of Spi-1 in the proerythroblast contributes to leukemic transformation by blocking the erythroid differentiation (Moreau-Gachelin et al., 1996). These findings indicate that additional genetic alterations are required to confer proliferative and survival advantages to the erythroblast, but direct evidence has been lacking. One approach to address the question of events cooperating for leukemic transformation in the *spi-1*-TG erythroblasts was to study the intracellular signaling pathways in HS2 cells. We previously demonstrated that the PI3-kinase/Akt and the PKC/Erk1/2 pathways were activated in HS2 cells and that these two signaling pathways cooperated to induce survival and proliferation of *spi-1*-TG proerythroblasts in the absence of Epo (Barnache et al., 2001). These data raised the question of the nature of the molecular events responsible for such signaling dysregulations.

Cell growth and survival pathways are linked to multiple signaling cascades through tyrosine phosphorylation processes. Altered or elevated tyrosine kinase activities arising from mutations or protein structure abnormalities have been implicated in malignant hematopoiesis. A well-known example is the constitutive tyrosine kinase activity of the Bcr-Abl oncoprotein expressed in human chronic myelogenous leukemia (Shtivelman et al., 1985). Many mutations in receptor tyrosine kinases are linked to both acute and chronic leukemia. For example, somatic mutations in FLT-3 (FMS-like tyrosine kinase) are frequently detected in AML (Abu-Duhier et al., 2000; Yamamoto et al., 2001) and myelodysplasia (Horiike et al., 1997), whereas somatic mutations in the Kit receptor for stem cell factor (SCF) are mainly associated with mastocytosis (Pignon et al., 1997; Tsujimura et al., 1996) and AML (Beghini et al., 2000; Gari et al., 1999).

Here, we report the presence of acquired mutations in the *Kit* gene in 86% of tumors isolated during the erythroleukemic progression in *spi-1*-TG mice. Mutations affected mainly codon 814, and occasionally codon 818, and conferred a ligand-independent tyrosine kinase activity to Kit. We demonstrated that ectopic expression of either one Kit mutant in HS1 cells conferred Epo independence in vitro and tumorigenicity in vivo to HS1 cells. Using PP1, PP2, and imatinib mesylate as pharmacological inhibitors of Kit tyrosine kinase activity, we also show that the growth factor-autonomous expansion of HS2 cells induced by Kit mutants occurred via the activation of the PI3K/Akt and MAPK signaling pathways. Altogether, these findings indicate an essential role for Kit mutations in the progression of erythroleukemia in *spi-1*-TG mice.

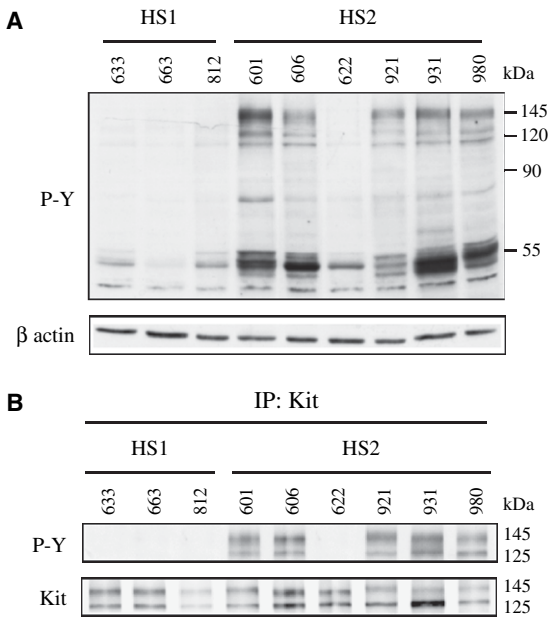

Figure 1. Expression and tyrosine phosphorylation of Kit in HS1 and HS2 cells

A: Whole-cell extracts prepared from the indicated HS1 and HS2 cells were analyzed by Western blotting using anti-phosphotyrosine (P-Y) antibodies. This blot was stripped, and an anti-actin antibody was used to visualize the equal loading of proteins (bottom panel). Molecular weight standards are indicated on the right.

B: Protein extracts from the indicated HS1 and HS2 cells were immunoprecipitated (IP) with anti-Kit antibody. The immunoprecipitates were analyzed by Western blotting with anti-P-Y antibodies. This blot was then stripped, and the anti-Kit antibody was used to verify Kit immunoprecipitation. Three independent experiments gave the same results.

Results

Kit is constitutively activated in tumorigenic HS2 cells from *spi-1*-TG mice

We previously reported that the MAPK and PI3K pathways were constitutively activated in Epo-independent and tumorigenic *spi-1*-TG proerythroblasts (HS2 cells) (Barnache et al., 2001). To approach the causes leading to constitutive activations of these signaling pathways, we performed a comparative analysis of the pattern of proteins phosphorylated on tyrosine in three HS1 cell lines and six HS2 cell lines derived from enlarged spleen of mice during the early (HS1) and late (HS2) stages of the leukemia. Western blotting of whole-cell extracts with an antibody specific for phosphotyrosine residues (anti-P-Y antibody) showed that both the number and the level of tyrosine-phosphorylated proteins in five HS2 cell lines were strongly increased in comparison to HS1 cells (Figure 1A). One prominent difference was the detection of 130–140 kDa tyrosine-phosphorylated proteins, which were present in HS2 cells but absent in HS1 cells. One possible candidate was the SCF receptor Kit. Confirmation of this assumption was obtained by immunoprecipitation of cell lysates with an anti-Kit antibody followed by an anti-phosphotyrosine immunoblotting. Kit was detected as two isoforms (125 and 145 kDa) in both HS1 and HS2

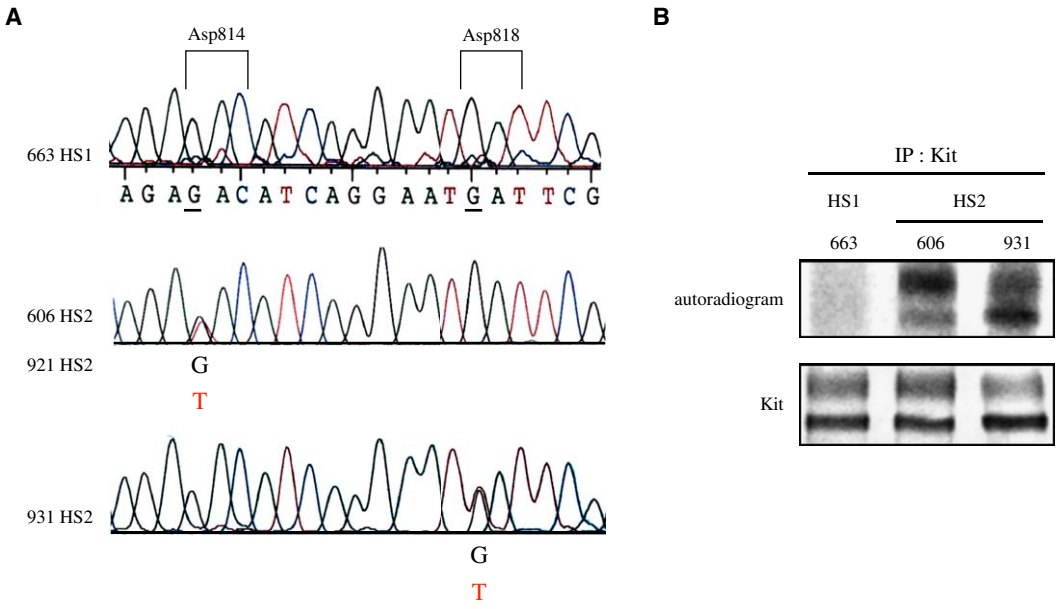

Figure 2. Genomic sequencing of the Kit gene at exon 17 and kinase activity of Kit mutants

A: Point mutations affect codons 814 and 818 of Kit. The nucleotide sequence of the relevant portion of *Kit* exon 17 from 663 HS1 spleen, 606 and 921 HS2 spleen tumor, and 931 HS2 spleen tumor is shown. In 663 HS1 cells, the gene is wild-type, and the nucleotide affected by mutation in 606, 921, or 931 HS2 tumor is underscored. In the 606 HS2 tumor, the mutation G→T at codon 814 changes Asp to Tyr. In the 931 HS2 tumor, G→T at codon 818 changes Asp to Tyr. As the wild-type sequence is also detected in both cases, the mutation is a heterozygous mutation.

B: Constitutive activation of Kit in HS2 cells. Kit was immunoprecipitated from indicated HS1 and HS2 cell lysates using the anti-Kit antibody. The Kit immunoprecipitates were incubated with [γ-^{32}P ATP], and the incorporated ^{32}P was visualized by autoradiography (upper panel) before immunoblotting with anti-Kit antibody (lower panel). Autoradiograms are representative of three experiments.

immunoprecipitates (Figure 1B). However, in contrast to HS1 cells, where Kit was not tyrosine phosphorylated, the receptor was phosphorylated on tyrosine in most HS2 cell lines. Thus, HS1 and HS2 cell lines express Kit, but tyrosine phosphorylation of the receptor is seen only in HS2 cells, suggesting that Kit could be activated in these cells.

Kit is mutated in tumorigenic HS2 cells

Two mechanisms of Kit activation in malignant cells are known: autocrine/paracrine stimulation of the receptor by its ligand SCF, or acquisition of activating mutations. HS2 cells did not produce detectable levels of SCF (our unpublished data), suggesting that autocrine or paracrine mechanisms were unlikely. Several mutations in either the juxtamembrane domain or the catalytic domain have been described that lead to ligand-independent activation of the Kit tyrosine kinase activity (Furitsu et al., 1993; Tsujimura et al., 1996). Thus, we sequenced the full-length *kit* transcripts in three HS2 cell lines (606, 921, and 931). In two of them (606 and 921), a single nucleotidic change G→T at position 2440 from the first nucleotide in the coding region was detected (Figure 2A) leading to the substitution of aspartic acid 814 for tyrosine (D814Y). In cell line 931, the D814Y mutation was not found, but we identified a G→T substitution at position 2452 in exon 17 changing aspartic acid 818 to tyrosine (D818Y). In the three cell lines, a wild-type *kit* cDNA was also sequenced, revealing that normal and mutated alleles were transcribed.

The 606, 921, and 931 cell lines were derived from the enlarged spleens of *spi-1*-TG mice during the HS2 phase of the disease. To assess whether the Kit mutations were already present in vivo, *Kit* exon 17 encoding the D814 and D818 cDNA region was sequenced from DNA extracted from the primary splenic tumors. The mutation G→T at position 2440 was identified on one allele, demonstrating that the mutation had occurred in vivo during the development of erythroleukemia. To search further for the occurrence of these mutations during progression of erythroleukemia in *spi-1*-TG mice, we sequenced *Kit* exon 17 from DNAs prepared from 25 additional HS2 splenic tumors. Data summarized in Table 1 show that the D814Y mutation was present in 18 out of 28 samples, while occurrence of the D818Y mutation was seen in two tumors. Rarely, mutations changed aspartic acid 814 to valine (D814V) or histidine (D814H) and aspartic acid 818 to valine (D818V). No mutation in *Kit* exon 17 was detected in the 622 HS2 cell line. This was in agreement with the fact that Kit was not tyrosine phosphorylated in extracts prepared from this cell line (Figure 1). It should be noted that 622 cells were previously characterized for Epo autocriny, which accounted for growth autonomy (Moreau-Gachelin et al., 1996). All of these leukemia retained the normal

Table 1. Kit mutations in HS2 tumors

Codon		814			818	
Nucleotide	wt	GAC			GAT	
	mut	TAC	GTC	CAC	TAT	GTT
Amino acid	wt	D			D	
	mut	Y	V	H	Y	V
HS2 sample number	28	18	2	1	2	1

Mutations (mut) were identified in 24 spleen tumors among 28 tested. Point mutations affect codons 814 and 818, which are both in exon 17. The wild-type (wt) allele was sequenced in each tumor.

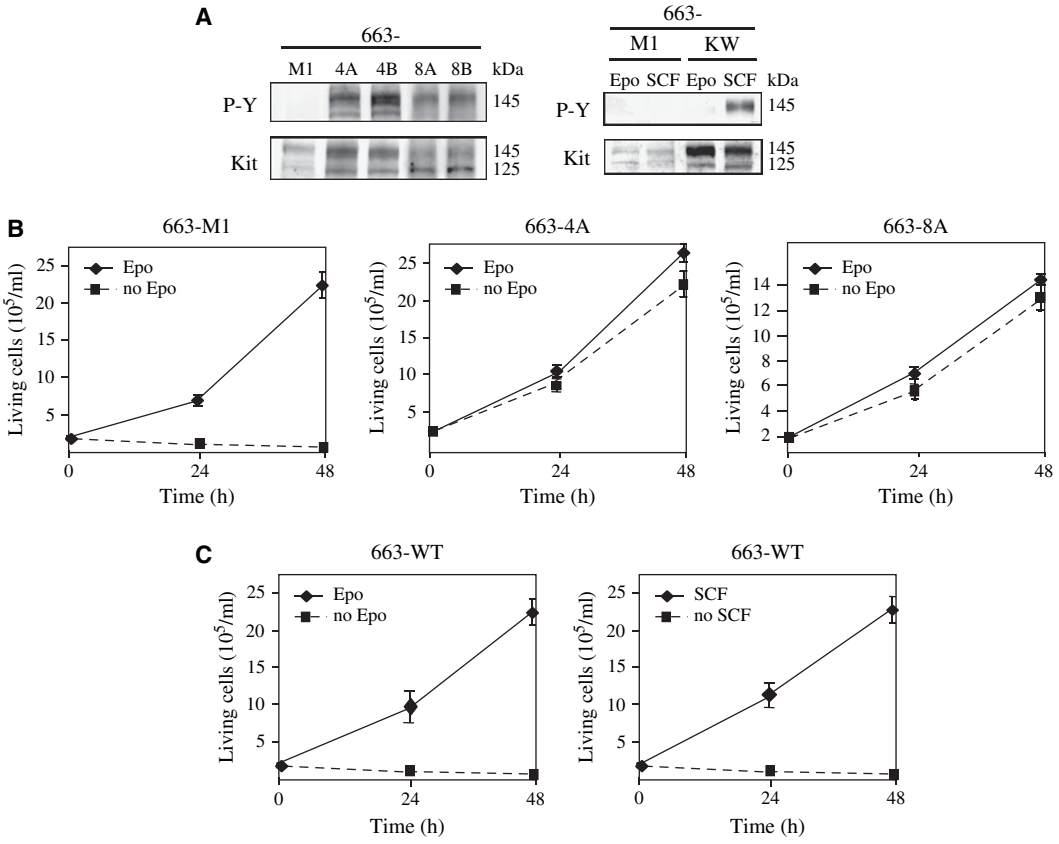

Figure 3. Enforced expression of Kit^{D814Y} and Kit^{D818Y} in HS1 cells confers Epo independence and tumorigenicity

A: Expression of Kit mutants in whole lysates from 663 HS1 cells transfected with either pEF-BOS and pMSCV-NeoR (M1), or pEF-BOS-Kit^{D814Y} and pMSCV-NeoR (4A and 4B), or pEF-BOS-Kit^{D818Y} and pMSCV-NeoR (8A and 8B), or pEF-BOS-Kit^{WT} and pMSCV-NeoR (KW) cultured in the presence of Epo or SCF. Lysates were analyzed by Western blotting using anti-P-Y antibodies. The same membrane was reprobed with anti-Kit antibodies.

B: 663 HS1 cells transfected with expression vectors for Kit^{D814Y} (663-4A cells) or Kit^{D818Y} (663-8A cells) or empty vector (663-M1 cells) were cultured for the indicated times in the presence or absence of Epo.

C: 663 HS1 cells transfected with expression vectors for Kit^{wt} (663-wt cells) were cultured for the indicated times in the presence or absence of Epo or SCF. The mean number of living cells and standard deviations were determined from three experiments.

Kit allele. Finally, the *Kit* exon 17 nucleotide sequence was determined from six HS1 spleens, and no mutation was detected.

To confirm that G→T substitution at residues 814 and 818 of Kit were activating mutations, kinase activity of Kit was directly measured in immunoprecipitates from 606 and 931 HS2 cells using an in vitro assay with radiolabeled ATP. Autoradiograms (Figure 2B) show that Kit was phosphorylated in extracts from both mutated cell lines, whereas phosphorylated forms of Kit were not detected in extracts from 663 HS1 cells. Altogether, these data show that both Kit mutations in HS2 cells lead to a ligand-independent kinase activity of this receptor and suggest that the mutation of Kit is a recurrent genetic alteration occurring during the leukemic progression in *spi-1*-TG mice.

Expression of Kit^{D814Y} and Kit^{D818Y} mutants confers Epo independence and promotes tumorigenicity to HS1 *spi-1*-TG proerythroblasts

The identification of Kit mutations in 86% of HS2 tumors suggested that they could be a causal factor in the growth factor independence and/or tumorigenicity of HS2 proerythroblasts. To determine the phenotypic consequences related to the expression of Kit^{D814Y} or Kit^{D818Y} in leukemic cells, the expression of Kit mutants was enforced in Epo-dependent 663 and 633 HS1 cells. Results with both cell lines were similar, and only data with 663 cells are detailed.

Stable populations of 663 HS1 cells were generated with expression constructs for Kit^{D814Y} (663-4 cells) or Kit^{D818Y} (663-8 cells). Pools of G418-resistant cells were used to avoid effects of clonal selection and amplified in the presence of Epo. For each mutation, two independent pools (A and B) were retained for further study. 663 HS1 cells were also transfected with an expression construct for wild-type Kit (663-KW cells) or a neomycin resistance gene vector (663-M1) to serve as controls. Whole extracts from transfected cells were analyzed by Western blotting using the anti-phosphotyrosine antibody. Then, the blots were reprobed with the anti-Kit antibody. Tyrosine-phosphorylated proteins corresponding to Kit were detected in cells transfected with Kit^{D814Y}- or Kit^{D818Y}-expressing vectors (Figure 3A) but were absent in both control cells cultured in the presence of Epo. In the 663-KW cells, the phosphorylated forms of Kit were only detected when cells were cultured in the presence of SCF (Figure 3A).

We first examined whether the growth of the 663-4A and 663-8A cells was changed with regard to their Epo dependency. Epo starvation did not modify significantly the growth characteristics of these cells (Figure 3B). Similar results were obtained for

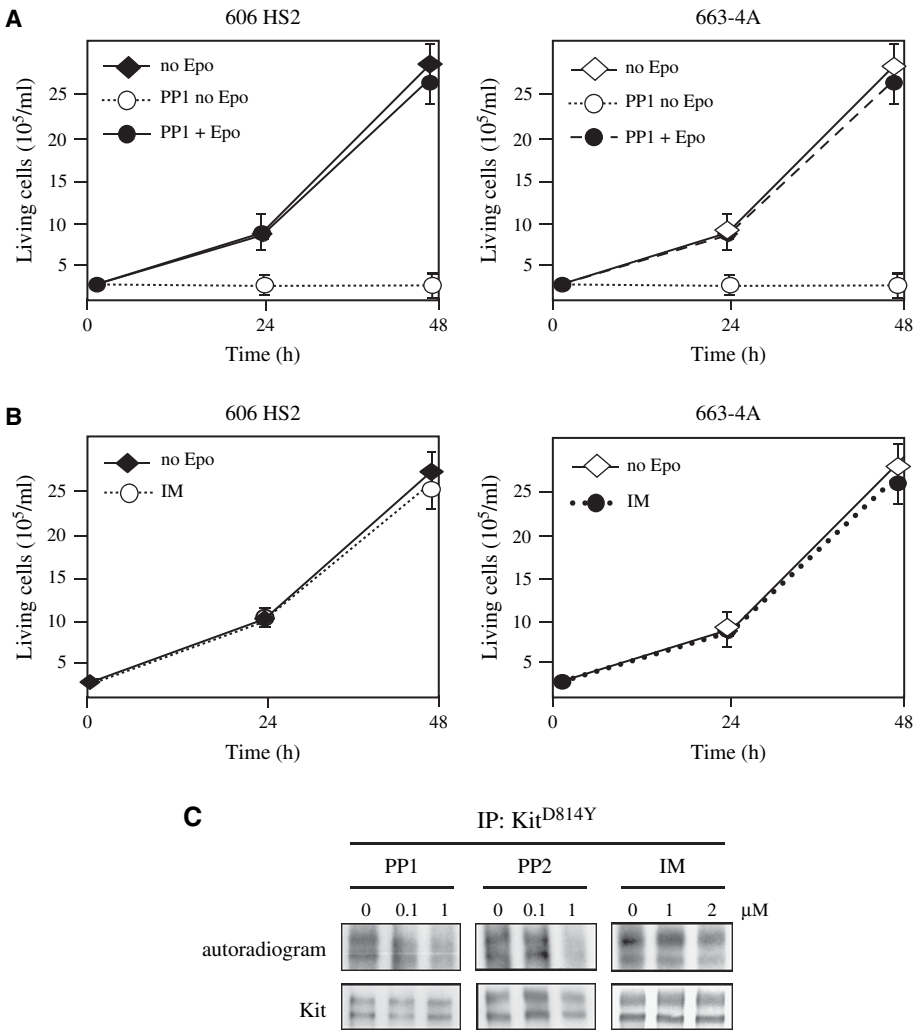

Figure 4. Effects of PP1 and imatinib mesylate on the proliferation of 606 HS2 cells and the kinase activity of Kit^{D814Y}

A: Proliferation of 606 HS2 cells harboring the Kit D814Y mutation and 663 HS1 cells transfected with expression vectors for Kit^{D814Y} (663-4A cells) in the presence of PP1 (4 μM) and in the presence (1 U/ml) or in the absence of Epo.

B: Proliferation of 606 HS2 cells and 663-4A cells in the presence of imatinib mesylate (IM; 0.75 μM) and in the absence of Epo. Viable cells were counted at indicated times. The mean number of living cells and standard deviations were determined from three experiments.

C: In vitro sensitivities of the kinase activity of Kit^{D814Y} to PP1, PP2, and IM. Kit was immunoprecipitated from 606 HS2 cell lysates using the anti-Kit antibody. The Kit immunoprecipitates were treated or not with the various inhibitors at the indicated concentrations and subsequently subjected to an autophosphorylation reaction in the presence of [γ-^{32}P ATP]. Kit autophosphorylation was visualized by autoradiography (upper panel). Receptor loading was controlled by immunoblotting with anti-Kit antibodies (lower panel). Autoradiograms are representative of three experiments.

663-4B and 663-8B cells (data not shown). In contrast, control NeoR-transfected 663-M1 cells remained unable to proliferate in the absence of Epo. With regard to 663-wt cells overexpressing Kit^{wt}, their growth was strictly dependent on either Epo or SCF (Figure 3C). These results demonstrate that the enforced expression of a mutated form of Kit (Kit^{D814Y} or Kit^{D818Y}) abolishes the Epo requirement of HS1 cells for growth and survival.

Next, the oncogenic potential of 663-4 or 663-8 cells (pools A and B) expressing Kit^{D814Y} or Kit^{D818Y} was assessed by subcutaneous injection of cells into immunodeficient mice, since recipients having a genetic background compatible with that of *spi-1*-TG mice were lacking. Within 2 weeks, all nude recipients (seven per cell line) developed solid tumors (0.5–1.5 cm diameter) at the injection site. Tumor efficiencies (100%) and size were similar to those observed after engraftment of HS2 cells (606 and 931). NeoR-expressing cells (663-M1 cells) as well as 663 HS1 cells failed to develop tumors. *Kit* exon 17 from DNAs extracted from subcutaneous tumors was sequenced. The expected mutations were detected, demonstrating that tumors derived from the injected cells (data not shown). Thus, ectopic expression of Kit^{D814Y} or Kit^{D818Y} confers tumorigenicity to HS1 cells.

Proliferation of $HS2^{Kit\ D814Y}$ cells is inhibited by PP1 and PP2 but not by imatinib mesylate

To address more directly the role of Kit mutations found in HS2 cells, we used three different pharmacological inhibitors of Kit kinase activity. PP1 and PP2 are compounds that inhibit several tyrosine kinases, including both the wild-type and mutated forms of Kit (Tatton et al., 2003; Zermati et al., 2003). 606 HS2 cells harboring the Kit^{D814Y} mutation and 663-4A cells expressing an ectopic Kit^{D814Y} were grown in the presence of either PP1 or PP2. Addition of PP1 (4 μM) to the culture medium completely inhibited cell growth, but the inhibition was not observed in the presence of Epo, ruling out a nonspecific cytotoxic effect of PP1 on the cells (Figure 4A). Similar data were obtained with PP2 used at an identical concentration and with 663-4B Kit^{D814Y}-transfected cells (data not shown). Imatinib mesylate (also called STI571 or Gleevec) is a compound shown to inhibit the kinase activity of a wild-type Kit (Heinrich et al., 2000), but not a Kit mutated on residue 816 in human (Ma et al., 2002; Zermati et al., 2003). Human codon 816 is the equivalent of codon 814 in the mouse. In contrast to data obtained with PP1 and PP2, the proliferation of 606 or 663-4 cells was not significantly affected by the addition of imatinib mesylate (0.75 μM)

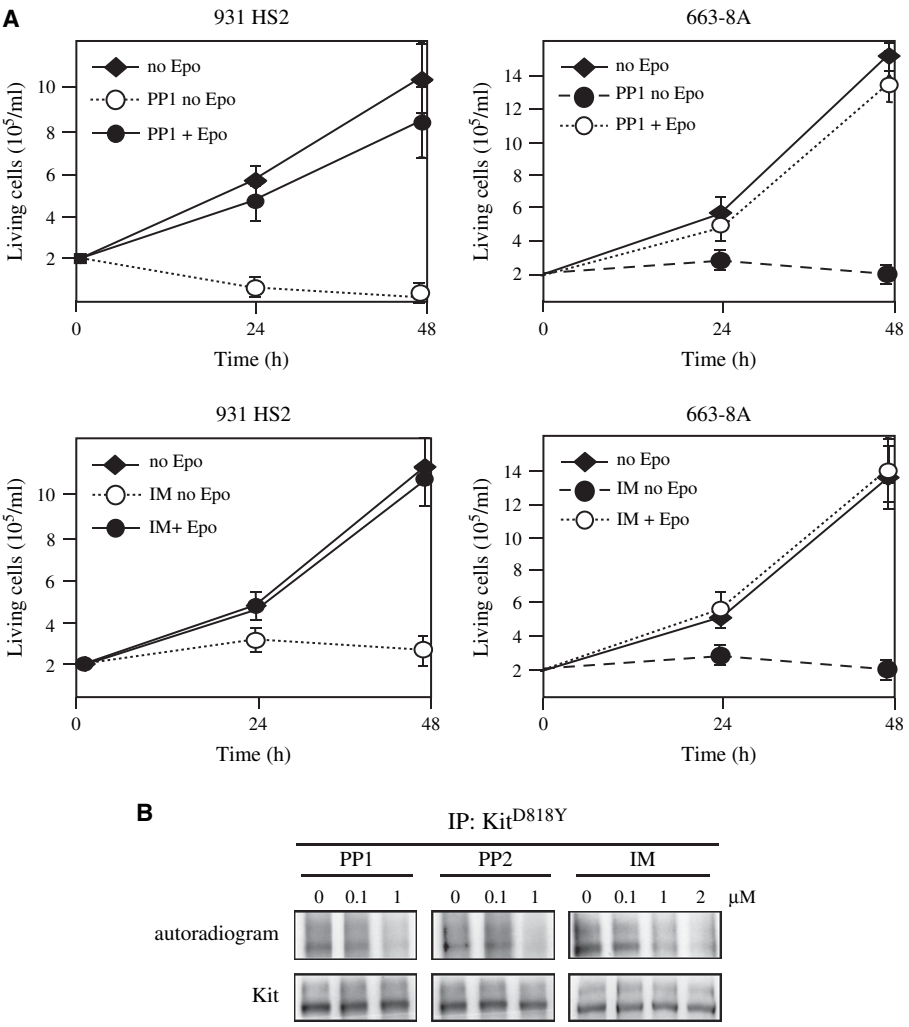

Figure 5. Effects of PP1 and imatinib mesylate on the proliferation of 931 HS2 cells and the kinase activity of Kit^{D818Y}

A: Proliferation of 931 HS2 cells harboring the Kit D818Y mutation and 663 HS1 cells transfected with expression vectors for Kit^{D818Y} (663-8A cells) in the presence of PP1 (4 μM) or imatinib mesylate (IM; 0.75 μM) and in the presence (1 U/ml) or in the absence of Epo. Viable cells were counted at indicated times. The mean number of living cells and standard deviations were determined from three experiments.

B: In vitro sensitivities of Kit^{D818Y} to PP1, PP2, and IM. Kit was immunoprecipitated from 931 HS2 cell lysates with anti-Kit antibodies. Kit immunoprecipitates were treated or not with the various inhibitors at the indicated concentrations and subsequently subjected to an autophosphorylation reaction in the presence of [γ-^{32}P ATP]. Kit autophosphorylation was visualized by autoradiography (upper panel). Receptor loading was controlled by immunoblotting with anti-Kit antibodies (lower panel). Autoradiograms are representative of three experiments.

(Figure 4B). Similar results were obtained when the expression of Kit^{D814V} was enforced in 663 or 633 HS1 cells.

Next, the effects of PP1, PP2, or imatinib mesylate on Kit kinase activity were evaluated in autophosphorylation kinase assays performed with Kit immunoprecipitates prepared from 606 HS2 cell extracts. Reprobing with the anti-Kit antibody demonstrated equivalent amounts of Kit in each immunoprecipitate. Addition of increasing concentrations of PP1 and PP2 to the kinase assay impeded the autophosphorylation of Kit^{D814Y} in a dose-dependent manner, while no effect was seen when imatinib mesylate was added at increasing concentrations up to 2 μM (Figure 4C). Thus, 606 HS2 cells carrying the Kit^{D814Y} mutation and 663-4A cells expressing an ectopic Kit^{D814Y} are equally responsive to the growth inhibitory effects of PP1 and PP2, and the inhibition of cell proliferation is correlated to the biochemical effects of PP1 and PP2 on the constitutive Kit kinase activity. In contrast, these cells are not affected by imatinib mesylate, which is unable to inhibit Kit^{D814Y} tyrosine kinase activity in vitro.

Proliferation of HS2$^{Kit\ D818Y}$ cells is inhibited by PP1, PP2, and imatinib mesylate

Similarly, we examined the effects of PP1, PP2, and imatinib mesylate on the growth of 931 HS2 cells that harbor the D818Y mutation in Kit and on the growth of 663-8A cells expressing an ectopic Kit^{D818Y}. In contrast to cells with the D814Y mutation, imatinib mesylate (0.75 μM) inhibited the autonomous growth of each cell type as did PP1 (4 μM) and PP2 (data not shown) (Figure 5A). Comparable results were obtained with 663-8B cells (data not shown). Inhibition of cell proliferation was abrogated when Epo was present in the culture medium, confirming that PP1, PP2, and imatinib mesylate targeted specifically the Epo-independent proliferation of HS2 cells. In addition, these drugs exerted a dose-dependent inhibitory effect on the constitutive kinase activity of Kit^{D818Y} when added to the in vitro kinase assay performed with Kit immunoprecipitates from 931 HS2 cells (Figure 5B). Thus, PP1, PP2, and imatinib mesylate inhibit the constitutive kinase activity of Kit^{D818Y} as well as arresting the autonomous proliferation of both 931 HS2 cells and 663-8A cells.

The MAPK and PI3K/Akt pathways are activated downstream from Kit^{D814Y} and Kit^{D818Y}

MAPK and PI3K/Akt pathways are constitutively activated in HS2 cells (Barnache et al., 2001). Thus, it could be hypothesized that the expression of Kit mutants in HS2 cells may mediate these constitutive signaling activations. We first investigated whether the activations of MEK/Erk1/2 and PI3K/Akt pathways

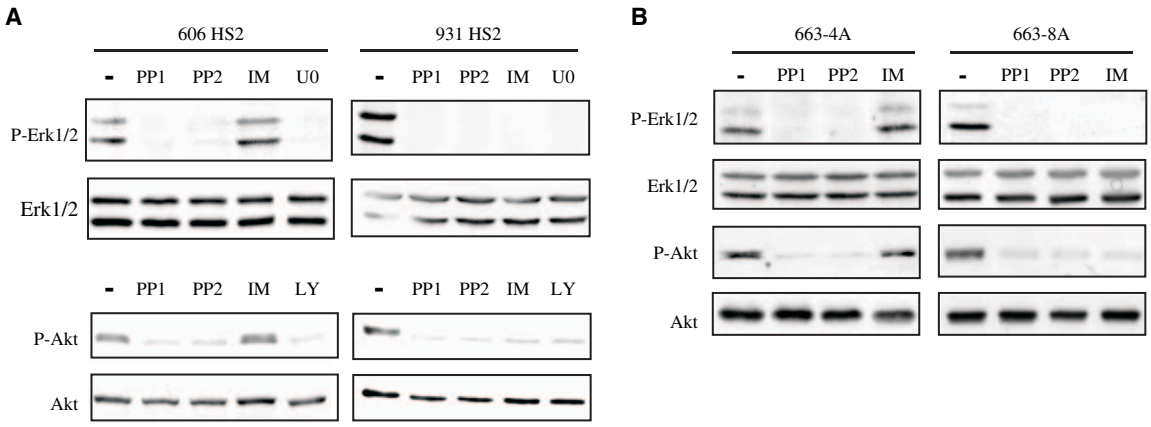

Figure 6. Effects of PP1, PP2, and imatinib mesylate on the activation of Erk1/2 and Akt

A: 606 and 931 HS2 cells were treated or not (−) for 2 hr with PP1 (4 μM), PP2 (4 μM), IM (0.75 μM), U0126 (U0; 20 μM), or LY294002 (LY; 10 μM).
B: 663 HS1 cells transfected with expression vectors for Kit^{D814Y} (663-4A cells) or Kit^{D818Y} (663-8A cells) were treated or not (−) for 2 hr with PP1, PP2, or IM. Whole-cell extracts were subjected to immunoblotting with antibodies indicated on the left of the panels. Western blots are representative of three independent experiments.

were sensitive to PP1, PP2, and imatinib mesylate in 606 and 931 HS2 cells. We measured the total and phosphorylated levels of Erk1/2 and Akt with appropriate antibodies in whole-cell extracts. Western blots in Figure 6A show that the constitutive phosphorylations of Erk1/2 or Akt were totally abolished in cell lines cultured in the presence of both PP1 (4 μM) or PP2 (4 μM) and the MEK inhibitor U0126 (20 μM) or the PI3K inhibitor LY294002 (10 μM). Comparable results were obtained with 663 HS1-transfected cells expressing either Kit^{D814Y} (663-4A cells) or Kit^{D818Y} (663-8A cells) in Figure 6B or Kit^{D814V} (data not shown). These data indicate that the constitutive activation of both Erk1/2 and PI3K/Akt pathways requires signaling inhibited by PP1 and PP2.

Finally, the effects of imatinib mesylate (0.75 μM) on the constitutive activation of MEK/Erk and PI3K/Akt pathways were compared in Kit^{D814Y}- versus Kit^{D818Y}-expressing cells (Figure 6). Both Erk1/2 and Akt phosphorylations were abolished by imatinib mesylate treatment in cells expressing the D818Y mutation (931 HS2 and 663-8A cells). In contrast, no reduction in phosphorylation of Erk1/2 or Akt was detected in cells expressing Kit^{D814Y} (606 HS2 and 663-4A) after treatment with this compound. The same results were observed for Kit^{D814V}-expressing cells (data not shown). Thus, the ability of imatinib mesylate to inhibit the constitutive activation of the Erk1/2 and Akt pathways depends on the mutation on Kit and is strictly correlated to the effects of this drug on cell growth. Altogether, these data demonstrate that mutations on Kit in malignant HS2 proerythroblasts are correlated to the activation of downstream Erk1/Erk2 and Akt signaling pathways.

SCF and Epo cooperate to sustain the proliferation of HS1 cells

Our initial studies failed to detect a significant growth dependence of *spi-1*-TG proerythroblasts on cytokines other than Epo (Moreau-Gachelin et al., 1996). The demonstration that SCF receptors were expressed in HS1 cells raised the question of a potential role for this receptor in HS1 cells. Accordingly, we reevaluated the sensitivity of HS1 cells to SCF. Figure 7A shows that SCF alone (100 ng/ml) could sustain the growth of 663 HS1 cells, though with a poor efficiency (even at higher concentrations), since cell numbers were 50% reduced at 48 hr when compared to cells grown with Epo (1 U/ml). This SCF-dependent growth was related to an activation of Kit, since a tyrosine-phosphorylated form of Kit was detected in immunoprecipitates from 663 HS1 cells stimulated by SCF, whereas it was absent in Epo-stimulated cells (Figure 7B). It was also related to the phosphorylation of Erk1/2 and Akt, although Erk1/2 activation was lower in 663 HS1 cells cultured in the presence of SCF than in those cultured in the presence of Epo (Figure 7C). With regard to 663KW-cells that overexpress Kit^{wt}, Epo or SCF induced a similar cell proliferation (Figure 3C) as well as equivalent levels of Erk1/2 and Akt activations (Figure 7D). Moreover, PP1 (4 μM) and imatinib mesylate (0.75 μM) abolished these SCF-dependent processes (Figures 7D and 7E). This indicated that SCF, like Epo, was able to trigger proliferative signals leading to Erk1/2 and Akt activations in HS1 cells.

Finally, we investigated whether SCF could cooperate with Epo to induce 663 HS1 cells to proliferate. Cells were cultured in the presence of a combination of SCF (100 ng/ml) and/or a suboptimal dose of Epo (0.05 U/ml). The growth of 663 HS1 cells was significantly reduced in the presence of a low Epo concentration (0.05 U/ml) or with SCF alone (100 ng/ml), as compared the optimal growth obtained with Epo at 1 U/ml. However, when SCF (100 ng/ml) was combined with Epo (0.05 U/ml), cell growth was restored to optimal levels obtained with Epo at 1 U/ml (Figure 7F). Similar results were observed with two other HS1 cell lines (data not shown). This demonstrates that SCF is not an optimal growth factor by itself for *spi-1*-TG proerythroblasts but can cooperate with Epo to drive the proliferation of HS1 cells, a finding that further supports a role for SCF in the expansion of HS1 cells in vivo.

Discussion

The erythroleukemia in *spi-1/PU.1* transgenic mice evolves as a multistep process (Moreau-Gachelin et al., 1996). Within about 4 months after birth, mice develop a severe anemia with massive infiltration of the spleen by erythroid precursor cells blocked at

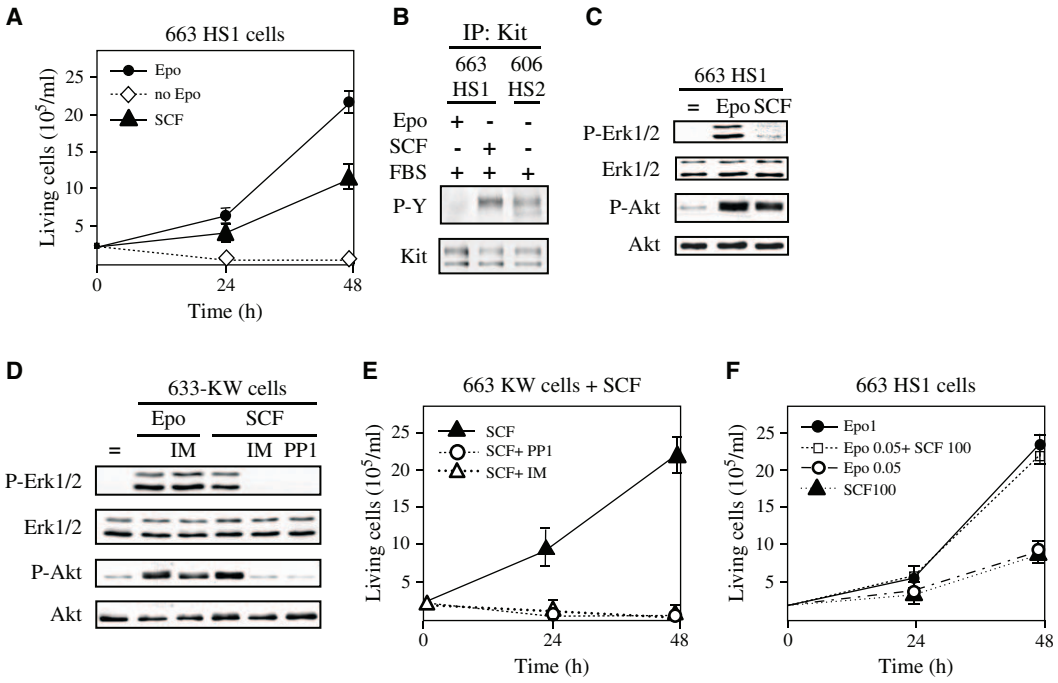

Figure 7. Cooperative effects of SCF and Epo to sustain the proliferation of HS1 cells

A: Growth of 663 HS1 cells when cultured in the presence of Epo (1 U/ml) or SCF (100 ng/ml) or in the absence of Epo and SCF for the indicated times.

B: 663 HS1 cells were starved in serum and Epo for 4 hr before being stimulated (+) 10 min with Epo (10 U/ml) or SCF (100 ng/ml) and FBS (10%). Kit immunoprecipitates were analyzed by Western blotting using anti-phosphotyrosine (P-Y) antibodies followed by anti-Kit antibodies. As control, Kit immunoprecipitates from 606 HS2 cells cultured in the absence of Epo and SCF were analyzed on the same Western blot.

C: Whole-cell extracts from 663 HS1 cells cultured in the presence of Epo (1 U/ml) or SCF (100 ng/ml) or deprived in Epo and SCF (=) were subjected to immunoblotting with antibodies indicated on the left of the panels.

D: Whole-cell extracts from 663-KW cells deprived in Epo and SCF (=) or cultured in the presence of Epo or SCF and treated or not for 1 hr with PP1 or IM were subjected to immunoblotting with antibodies indicated on the left of the panels.

E: 663-KW cells were cultured in the presence of SCF (100 ng/ml) and in the presence or in the absence of PP1 (4 μM) or IM (0.75 μM).

F: Growth of 663 HS1 cells cultured in the presence of Epo (1 U/ml or 0.05 U/ml) or SCF (100 ng/ml) or both Epo (0.05 U/ml) and SCF (100 ng/ml). The mean number of living cells and standard deviations were determined from three experiments.

a proerythroblastic stage. These preleukemic cells remain dependent upon Epo for their proliferation and are unable to produce tumors when transplanted into secondary hosts (HS1 stage). In a second stage, fully malignant proerythroblasts emerge that are growth factors independent for proliferation and tumorigenic in vivo (HS2 stage). This multistage pathology indicates that overexpression of Spi-1 is directly involved in the erythroid differentiation blockade but is not sufficient to promote full malignancy.

With the aim of identifying oncogenic events involved during leukemia progression, we found monoallelic mutations in the *Kit* gene in 86% of the tumors isolated during the HS2 stage of the disease. These mutations were gain-of-function, conferring a ligand-independent tyrosine kinase activity to Kit. The relevance of *Kit* mutations in the erythroleukemic process was assessed through the analysis of phenotypic changes generated in HS1 cells by an ectopic expression of Kit mutants. These cells acquired an autonomous proliferation in vitro and a tumorigenic phenotype in vivo. Using pharmacological inhibitors of Kit, we demonstrate that Kit mutations released the proerythroblast from Epo dependency via the constitutive activation of the PI3K/Akt and Erk1/2 signaling pathways. Thus, mutations in *Kit* appear to be master oncogenic events in the progression of the proerythroblast toward malignancy.

Kit is a class III tyrosine kinase receptor that belongs to the same subfamily as the receptors for PDGF, CSF-1, and FLT3 ligands. It consists of an extracellular domain with five immunoglobulin-like domains, a single transmembrane domain, and an intracytoplasmic kinase domain divided into an ATP binding region and a phosphotransferase region (Qiu et al., 1988; Yarden et al., 1987). Kit activation is triggered by SCF binding, which induces receptor dimerization followed by activation of the intrinsic tyrosine kinase and receptor *trans*-phosphorylation on specific tyrosine residues (Rottapel et al., 1991). Subsequently, signaling pathways including MAPK, PI3K, and Src kinase pathways can be activated (Timokhina et al., 1998; Wandzioch et al., 2004). Different mutations, which induce the constitutive activation of Kit and lead to proliferative disorders, have been described in human. Such mutations are of two types. The first type comprises point mutations resulting in ligand-independent catalytic activity of Kit (Moriyama et al., 1996). Selective residues are affected in the phosphotransferase domain, such as the especially frequent mutation on residue 816 that converts Asp to a variety of amino acids (Val, Tyr, or His). Such mutations are found in patients with mastocytosis (Longley et al., 1996; Pignon et al., 1997) and AML (Beghini et al., 2000; Wang et al., 2005). Mutations of the second type are either point mutations or variable deletions in the

juxtamembrane domain of the receptor and were found in human gastrointestinal stromal tumors (Chen et al., 2003; Hirota et al., 1998). In mice, Kit mutations affecting D814 have been described in one mastocytoma cell line (Tsujimura et al., 1994) and in one stroma-independent variant of an erythroleukemic cell line (Leslie et al., 1998).

The implication of Kit mutants in the malignant phenotype of HS2 proerythroblasts was demonstrated through cellular effects of various pharmacological inhibitors on the Kit kinase activity. Imatinib mesylate inhibits both the wild-type Kit and the juxtamembrane Kit mutants (Akin et al., 2004; Heinrich et al., 2000). With regard to the Kit mutants in the phosphotransferase domain, their sensitivity to imatinib mesylate depends on the mutation (Ma et al., 2002; Zermati et al., 2003). Indeed, this drug neither altered the constitutive kinase activity of Kit^{D814Y} in vitro nor affected the growth of HS2 cells with a Kit^{D814Y} mutation. In contrast, imatinib mesylate inhibits the Epo-independent growth of HS2 cells harboring the Kit^{D818Y} mutation as well as the kinase activity of Kit^{D818Y} in vitro, strongly arguing for a prime role of Kit^{D818Y} in signaling for growth autonomy. Tatton et al. showed that PP1 and PP2 had significant activity against both the wild-type Kit and the D816 mutant (Tatton et al., 2003). Indeed, PP1 and PP2 abolished the tyrosine kinase activity of Kit^{D814Y}, and we established that they also inhibit the tyrosine kinase activity of Kit^{D818Y} in vitro. Consistent with these biochemical effects, PP1 and PP2 inhibited drastically the autonomous growth of HS2 cells or the growth of HS1 cells rendered growth factor independent by the exogenous expression of Kit^{D814Y} or Kit^{D818Y}. These data support a mechanistic link between the expression of activated Kit mutants and cellular responses to drugs. Kit mutants mediate the activation of Erk1/2 and Akt in HS2 cells as well as in HS1 cells ectopically expressing Kit^{D814Y} or Kit^{D818Y}. Thus, the activations of Erk1/2 and Akt downstream from Kit mutants are sufficient to explain the growth factor-autonomous expansion of HS2 cells. The molecular links involved in these signaling pathways are currently under investigation.

Kit is expressed in HS1 cells, raising the question of a role for SCF in favoring expansion of the erythroblastic compartment in the preleukemic phase. Epo and SCF are two critical regulators for erythropoiesis. In fetal livers from mice deficient in Epo or Epo-R, BFU-E and CFU-E do not mature into erythrocytes (Wu et al., 1995b). Loss-of-function mutations at the *w* locus, which encodes the *Kit* gene, or the *sl* locus, which encodes the *SCF* gene, result in particular in a depletion in erythroid precursors at the BFU-E/CFU-E transition (Chabot et al., 1988; Geissler et al., 1988). A cooperation of SCF and Epo in the maturation and proliferation of erythroid progenitor cells around the CFU-E stage is also well documented (Panzenbock et al., 1998; von Lindern et al., 2001). It is proposed that Kit activates Epo-R by a physical association leading to tyrosine phosphorylation, a mechanism that provides a molecular explanation for the cooperative effects of Epo and SCF on erythropoiesis (Wu et al., 1995a). The acute expansion of the erythroid compartment intending to compensate the deficit in differentiated red blood cells in the diseased *spi-1*-TG mice takes place in the presence of SCF in the bone marrow and splenic microenvironments. In addition, our present data showing that SCF cooperates efficiently with Epo to induce in vitro proliferation of HS1 cells strengthen the hypothesis that both Epo and SCF may account for the amplification of the HS1 cell compartment in vivo. In this context, activating mutations occurring on a gene involved in the expansion of preleukemic HS1 cells appear to be selection events capable of conferring a strong proliferative advantage leading to the emergence of malignant clones.

Because the high frequency of Kit mutations suggests a strong specificity with regard to the cooperation of Spi-1 and Kit for erythroleukemic progression, we investigated whether a constitutive activated form of Flt3, as frequently detected in human AML (Yamamoto et al., 2001), could also cooperate with Spi-1 during erythroleukemic progression. Indeed, the ectopic expression of a mutated Flt3 (D835Y) in HS1 cells renders these cells growth factor independent, suggesting that a cooperation of Spi-1 with activated tyrosine kinases other than Kit is possible (unpublished data). However, as expected from their erythroblastic nature (Adolfsson et al., 2005), Flt3 is not expressed in HS1 and HS2 cells. This reinforces our hypothesis that selection for Kit mutations in leukemic HS2 cells was related to the function of Kit in preleukemic HS1 cells.

Other oncogenic alterations were observed in HS2 tumors. Among the four HS2 tumors that did not harbor Kit mutations, two secreted Epo, indicating that Epo autocriny may be an alternative cooperative pathway (Moreau-Gachelin et al., 1996). Loss of function by mutations in the p53 gene have been identified in 60% of HS2 spleen tumors (Barnache et al., 1998). Kit mutations were detected with the same frequency in tumors with a p53 genotype that was either wild-type or mutated. Thus, p53 deficiency cannot be considered as a cooperating oncogenic event in this erythroleukemic process. Nevertheless, it cannot be excluded that other alterations than Kit mutation participate in in vivo progression from the HS1 to the HS2 stage.

Studies of oncogenic alterations in human AML led to the proposal of a "two-hit" model of leukemogenesis (Gilliland, 2001). In this model, an acute leukemia would arise from the cooperation between one class of mutations that interferes with differentiation and a second class of mutations that confers a proliferative advantage to cells. Indeed, in some human AML, combinations of dual mutations in the transcription factor AML1 and the kinase receptors FLT3 or Kit have been reported (Beghini et al., 2000; Matsuno et al., 2003; Wang et al., 2005) and are a poor prognostic factor (Care et al., 2003). Acute erythroleukemias in *spi-1*-TG mice as well as in Friend virus-infected mice add strong support to the concept that aberrations in transcription factors and tyrosine kinases are required for overt leukemia. In the Friend model, the SFFV-infected erythroleukemic cells are blocked in their differentiation as a consequence of *Spi-1* overexpression. Their growth and survival no longer depend on Epo because of the activation of Epo-R by the viral gp55 (Muszynski et al., 1998; Nishigaki et al., 2000), alleviating the need for selection of an oncogenic signaling event. Indeed, no Kit mutation was detected in Friend tumor cells (our unpublished data). In the *spi-1*-TG mice, the overexpression of Spi-1 prevents erythroid differentiation, and the resulting anemia induces a compensatory expansion of the proerythroblastic compartment. Then, the *spi-1*-TG mice are predisposed to clonal disorders that affect the erythroid lineage. In most of the cases, Kit mutations appear as the second and crucial hit that is associated with the progression of proerythroblasts toward malignancy. Thus, this model of leukemic transformation requires the collaboration of at least one proliferative and one differentiation blocking event. It underscores the similarities between mouse and human leukemias and appears as a fruitful experimental model to

dissect the respective roles of crucial genes affecting the leukemic process.

Experimental procedures

Transgenic mice and cell lines

The *spi-1*-TG mice were generated by germinal insertion of a *spi-1* transgene whose transcription is driven by the SFFV LTR. HS1 cells and HS2 cell lines were established from bone marrow and enlarged spleen of leukemic mice as described previously (Moreau-Gachelin et al., 1996). Experiments on mice were performed in accordance with relevant institutional guidelines from the Direction Départementale des Services Vétérinaires de Paris (approval 16-08-2004). Cells were cultured in α minimum essential medium (αMEM; GibcoBRL) supplemented with 10% fetal calf serum (FCS; GibcoBRL) and 1 U/ml of recombinant human Epo (Cilag, Levallois-Perret, France) when indicated.

For growth kinetics, cells were plated at 2×10^5 cells/ml. LY294002 (PI3 kinase inhibitor; Sigma), U0126 (MEK inhibitor; Promega), PP1 (Biomol), PP2 (Calbiochem), and imatinib mesylate (Novartis Pharma, Basel, Switzerland) were added at the indicated concentrations in culture medium. Viable cell numbers were monitored by 0.2% of Trypan blue exclusion staining (Sigma-Aldrich).

Immunoprecipitation and Western blotting

Cells were lysed at 2×10^7 cells/ml in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 25 mM β-glycerophosphate, 1 mM Na pyrophosphate, 10% glycerol, 1% NP40, 1 mM Na_3VO_4, 1× protease inhibitor; Roche). Lysates were centrifuged at 18,000 × g for 15 min at 4ºC. Supernatants were incubated with Kit antibodies for 2 hr at 4ºC. The immunoprecipitates bound to protein G Sepharose (Amersham) were washed extensively with 1% NP40 lysis buffer and 0.1% NP40 lysis buffer. Whole-cell extracts or immunoprecipitates were fractionated by SDS-PAGE, blotted, and visualized by the enhanced chemiluminescence (ECL) detection system (Amersham) as previously described (Barnache et al., 2001). Primary antibodies included 4G10 anti-phosphotyrosine antibody (UBI); rabbit immune serum anti-Kit, anti-phospho-Akt (9271), anti-Akt (9272), anti-phospho-p44/p42 MAP kinase (9106), and anti-p44/42 MAP kinase (9102) (purchased from New England Biolabs, Beverly, MA); and anti-βactin (Sigma-Aldrich).

Kit sequencing

Kit cDNAs were synthesized with SuperScript II reverse transcriptase (Gibco BRL) and amplified by PCR with PFU DNA polymerase (Stratagene, LaJolla, CA). The nucleotidic sequence was determined from five independent RT-PCR amplifications. The exon 17 was amplified by PCR using PFU DNA polymerase, and PCR products were sequenced with the forward primer 5′-CCT TTTCTCCCCCAACATGT-3′ and the reverse primer 5′-TGGAGAAAGGTACT CACATT-3′.

Kit mutagenesis

The murine wild-type Kit expression vector (pEF-BOS-Kit^{wt}) was supplied by Dr. M. Mizuki. The Kit^{D814Y} and Kit^{D818Y} mutants into the pEF-Bos expression vector were generated by mutagenesis of the wild-type *Kit* cDNA using the QuikChange Site-Directed Mutagenesis System (Stratagene, LaJolla, CA).

Transfections

Fifty micrograms of the plasmids pEF-BOS, pEF-bos-Kit^{wt}, pEF-BOS-Kit^{D814Y}, pEF-BOS-Kit^{D818Y}, or LXSN-Kit^{D814V} (Casteran et al., 2003) and/or 5 μg of the selection vector pMSCV-NeoR (Clontech, Palo Alto, CA) were transfected by electroporation (240 V, 960 μF) using a BioRad gene pulser in 2×10^7 663 or 633 HS1 cells. Stable transfectants were selected in growth medium containing 800 μg/ml G418 (Invitrogen) in the presence of Epo (1 U/ml).

In vitro kinase assay

After incubation of Kit immunoprecipitates with protein G Sepharose beads (Pharmacia), the beads were pelleted and washed twice with lysis buffer. Next, beads were resuspended and washed in kinase buffer (20 mM PIPES, 10 mM $MnCl_2$). Kinase reaction was performed for 10 min at 20ºC in 30 μl of kinase buffer supplemented with [γ-^{32}P ATP] (10 μCi). Reactions were terminated by the addition of an equal volume of gel loading buffer. After SDS-PAGE, the gels were electrotransferred onto hybond nitrocellulose membrane (Amersham Pharmacia Biotech, UK), and the kinase reaction products were detected by autoradiography.

In vivo tumorigenicity of cells

Cells (10^7 cells/500 μl in αMEM medium containing 2% FBS) were injected by subcutaneous route into 8- to 10-week-old female nude mice. Tumor nodules were taken off when the tumor mass reached at least 0.5 cm in diameter.

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