Myeloproliferative stem cell disorders by deregulated Rap1 activation in *SPA-1*-deficient mice

Daisuke Ishida,[1] Kohei Kometani,[1] Hailin Yang,[1,7] Kiyokazu Kakugawa,[1] Kyoko Masuda,[1] Kazuhiro Iwai,[1,8] Misao Suzuki,[4] Shigeyoshi Itohara,[5] Tatsutoshi Nakahata,[2] Hiroshi Hiai,[3] Hiroshi Kawamoto,[6] Masakazu Hattori,[1] and Nagahiro Minato[1,*]

[1] Department of Immunology and Cell Biology, Graduate School of Biostudies
[2] Department of Pediatrics
[3] Department of Pathology
Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan
[4] Center for Animal Resources and Development, Kumamoto University, Kumamoto 862-0976, Japan
[5] Brain Science Institute, RIKEN, Saitama 351-0198, Japan
[6] Research Center for Immunology and Allergy, RIKEN, Yokohama 230-0045, Japan
[7] Present address: Department of Immunoregulation, Dana Farber Cancer Research Institute, Harvard Medical School, Boston, Massachusetts 02115.
[8] Present address: Department of Molecular Cell Biology, Osaka City University, Osaka 585-8585, Japan.
*Correspondence: minato@imm.med.kyoto-u.ac.jp

Summary

SPA-1 (signal-induced proliferation-associated gene-1) is a principal Rap1 GTPase-activating protein in hematopoietic progenitors. SPA-1-deficient mice developed a spectrum of myeloid disorders that resembled human chronic myelogenous leukemia (CML) in chronic phase, CML in blast crisis, and myelodysplastic syndrome as well as anemia. Preleukemic SPA-1-deficient mice revealed selective expansion of marrow pluripotential hematopoietic progenitors, which showed abnormal Rap1GTP accumulation. Overexpression of an active form of Rap1 promoted the proliferation of normal hematopoietic progenitors, while SPA-1 overexpression markedly suppressed it. Furthermore, restoring *SPA-1* gene in a SPA-1-deficient leukemic blast cell line resulted in the dissolution of Rap1GTP accumulation and concomitant loss of the leukemogenicity in vivo. These results unveiled a role of Rap1 in myeloproliferative stem cell disorders and a tumor suppressor function of SPA-1.

Introduction

Ras mutations are responsible for many types of human malignancy (Bos, 1988), and Rap1, a close member of Ras-family GTPases, has been discovered originally as a protein counteracting the functions of oncogenic K-Ras in a fibroblast cell line (Kitayama et al., 1989). Rap1 shows the highest homology to Ras among the family and shares several downstream effecter molecules with Ras, including Raf family kinases (Bos et al., 2001), and it was reported that Rap1GTP either attenuated Ras-mediated ERK activation via competitive interference with c-Raf-1 activation (Cook et al., 1993; Hu et al., 1997) or induced ERK activation directly via B-Raf activation (Vossler et al., 1997; York et al., 1998) depending on the types of model cell lines. On the other hand, evidence has been accumulated that Rap1 mediated the activation of integrins (Arai et al., 2001; Katagiri et al., 2000; Reedquist et al., 2000; Tsukamoto et al., 1999) and regulated various integrin-dependent cellular functions (Caron et al., 2000; Katagiri et al., 2002; Sebzda et al., 2002; Shimonaka et al., 2003).

Activation of Rap1 by extracellular signals is mediated by various kinds of guanine nucleotide exchange factors (GEF), including C3G regulated by receptor-associated protein tyrosine kinases (Gotoh et al., 1995), Epac directly regulated by cAMP (de Rooij et al., 1998), and CalDAG-GEF activated by Ca^{2+} and DAG (Kawasaki et al., 1998), while Rap1GTP is inactivated to Rap1GDP by specific GTPase-activating proteins (GAP). At present, two groups of Rap1GAPs sharing a catalytic GAP-related domain (GRD) are identified, rapGAP I, II (Mochizuki et al., 1999; Rubinfeld et al., 1991) and SPA-1 (signal-induced proliferation-associated gene-1) family proteins including SPA-1(Hattori et al., 1995; Kurachi et al., 1997), E6TP1 (Gao et

SIGNIFICANCE

Rap1 GTPase was isolated originally as a potential antagonist of oncogenic K-Ras over a decade ago. While several biological functions of Rap1 were revealed in cell line models since then including modulation of Ras-ERK signaling and activation of integrins, its possible involvement in malignancy remained unknown. By using the mice targeted for *SPA-1* gene encoding a Rap1-specific GTPase-activating protein, we demonstrate that deregulated activation of Rap1 in the hematopoietic stem cells results in a spectrum of myeloid disorders that resemble human CML in chronic phase, CML in blast crisis, and MDS. Here we indicate that abnormal Rap1 activation causes malignancy in vivo and uncovers the role of SPA-1 as a tumor suppressor in hematopoietic stem cells.

al., 1999), and SPAR (Pak et al., 2001), which appear to have different tissue distribution. In addition, tuberin, a product of tuberous sclerosis-2 gene in human, shows a partial homology to GRD and is reported to exhibit Rap1GAP activity (Wienecke et al., 1995). The Rap1GAPs plays a major role in the spatiotemporal control of intracellular Rap1 activation (Ohba et al., 2003). We reported that Rap1GAPs were bound to AF-6 (afadin), a fusion partner of ALL1 in human myeloid leukemia (Prasad et al., 1993), via a conserved GRD, through which the accessibility to Rap1GTP could be regulated (Su et al., 2003).

Several recent reports have suggested the involvement of dysregulated Rap1signaling in malignancy. It was reported that E6TP1 was targeted for degradation by oncogenic human papillomavirus E6 protein via E6AP ubiquitin ligase in epithelial cells (Gao et al., 2002), which highly paralleled the cellular transformation by E6 oncoprotein (Gao et al., 2001). It was indicated also that CalDAG-GEFI was activated recurrently by proviral insertion in leukemia-prone BXH-2 recombinant strain of mice (Dupuy et al., 2001). Most recently, it was reported that a subset of human cancer cell lines showed missense mutations of *DOCK4* gene that encoded a Rap1-activating protein regulating intercellular junctions, and the protein with a recurrent mutation was defective in Rap1 activation (Yajnik et al., 2003). In the present study, we report that SPA-1 is a principal GAP for Rap1 in hematopoietic progenitors, and null mutant mice of *SPA-1* gene (SPA-1$^{-/-}$) develop a spectrum of myeloid disorders that resemble human chronic myelogenous leukemia (CML) in chronic phase, CML in blast crisis, and myelodysplastic syndrome (MDS), unveiling a role of SPA-1 as a tumor suppressor of myeloid malignancy.

Results

SPA-1 is a principal Rap1GAP in the immature bone marrow cells

In the normal bone marrow cells (BMC), both types of Rap1-GAPs, SPA-1 and rapGAP, were expressed. Cell fractionation analysis, however, revealed that lineage marker-negative (lin$^-$) immature BMC almost exclusively expressed SPA-1, while lin$^+$ (Gr-1$^+$ Mac-1$^+$ Ter119$^+$) BMC contained rapGAP with marginal SPA-1 (Figure 1A). Rap1 expression was comparable between the two populations. We also examined an IL-3-dependent promyeloblastic cell line (32D), which could be induced to differentiate into mature granulocytes in the presence of G-CSF. As also shown in Figure 1A, 32D cells growing in IL-3-containing medium expressed SPA-1 with marginal rapGAP. After the shift to G-CSF-containing medium, the cells gradually lost SPA-1 expression (day 3), and the maturated and growth-arrested granulocytes exhibited undetectable SPA-1 with significant rapGAP instead (day 5). These results indicated that expression of Rap1GAP species in immature myeloid cells was shifted from SPA-1 to rapGAP according to their maturation and that SPA-1 was a principal Rap1GAP in the immature hematopoietic progenitors. To explore the roles of SPA-1 in vivo, we generated *SPA-1* gene-targeted mice by homologous recombination, replacing exons 5 to 8 encoding GRD with a *neo*-containing targeting vector (Figure 1B). Mice homozygous for the mutant allele (SPA-1$^{-/-}$) expressed no detectable 130 kDa SPA-1 protein, while heterozygous mice (SPA-1$^{+/-}$) exhibited roughly half a level of SPA-1 showing a gene-dosage effect (Figure 1B). Expression of its substrate Rap1 was unaffected by the SPA-1 mutation.

Development of a spectrum of myeloid disorders in SPA-1-deficient mice

SPA-1$^{-/-}$ mice were born and developed normally. After around a year, however, all the SPA-1$^{-/-}$ mice started to reveal abnormal peripheral blood (PB) leukocyte numbers and anemia (Figure 2A). The mice were sacrificed for autopsy when they developed life-threatening anemia of less than 6×10^6 RBC/mm^3 or leukocytosis over 40×10^3 cells/ mm^3, or when they apparently became sick. In 14 out of the total 60 SPA-1$^{-/-}$ mice of the present cohort (group I), PB leukocytes were increased, which were predominated by mature granulocytes with few blast cells (Figure 2B). These mice exhibited marked splenomegaly with extensive extramedullary hematopoiesis of all the lineages including abundant erythroblasts (Figures 2A and 2B). BMs were pale, hypercellular, and predominated by maturated myeloid cells with markedly reduced erythroid component (Figure 3A). These features resembled human CML in chronic phase (cp). A minor group (group II, eight mice) showed pancytopenia with dysplastic granulocytes, large mono- or binuclear megakaryocytic cells, and some blastic cells in the PB (Figures 2A and 2B). The spleens were enlarged albeit less markedly, and the BMs often revealed mosaic hyperplastic and hypoplastic regions, reminiscent of human MDS (Figure 3A). The rest (group III, 35 mice) exhibited peripheral leukocytosis containing varying proportions (14% to 85%) of blast cells (Figures 2A and 2B). The blast cells were of diverse lineages including myeloid, erythroid, B and mixed lineages (Mac-1$^+$B220$^+$) except for T lineage, and extensively infiltrated in BM and other vital organs (Figures 2B, 3A, and 3B). Southern blot analysis for immunoglobulin heavy chain gene indicated that at least B220$^+$ blast cells were mono- or oligoclonal (Figure 3C). Overall, 95% of SPA-1$^{-/-}$ mice developed these myeloid disorders by 16 months. Transfer of the spleen cells from a group I mouse into SCID mice induced essentially identical cp-CML-like disease, and that of either PB or BMC from a group III mouse caused marked increase in the blast cells with heavy infiltration into vital organs within 2 months (Table 1). It was noted that one recipient of the group III PB developed cp-CML-like disease. While SPA-1$^{+/+}$ littermates (50 mice) showed no evidence of myeloid disorders until 20 months, 15% of SPA-1$^{+/-}$ littermates (16 out of 102 mice) developed abnormal hematological findings (the mean WBC, $22.3 \pm 5.8 \times 10^3$ cells/mm^3 and RBC, $6.35 \pm 0.6 \times 10^6$ cells/mm^3 versus $8.7 \pm 0.04 \times 10^3$ cells/mm^3 and $9.31 \pm 0.04 \times 10^6$ cells/mm^3 in SPA-1$^{+/+}$ mice) with splenomegaly, among which four mice showed CML in chronic phase and the rest CML in blast crisis.

Increase in pluripotent hematopoietic progenitors in SPA-1-deficient mice

We first examined the numbers of committed hematopoietic progenitors (CFU-C) in SPA-1$^{-/-}$ mice with cp-CML. As shown in Figure 4A, frequencies of CFU-C of all the lineages were increased in particular GM-CFU-C in the BMs and E- and Mix-CFU-C in the spleens with average colony sizes being largely unchanged. Since total nucleated cell numbers in the spleens and BMs were increased up to 50 times and 1.5 to 2 times, respectively, the overall CFU-C were estimated to reach nearly 100 times of the control mice. Furthermore, significant numbers of E- and Mix-CFU-C were detected in the circulation of SPA-1$^{-/-}$ mice, suggesting either premature dislodgment or "spill-over" of the expanded progenitors. We then examined

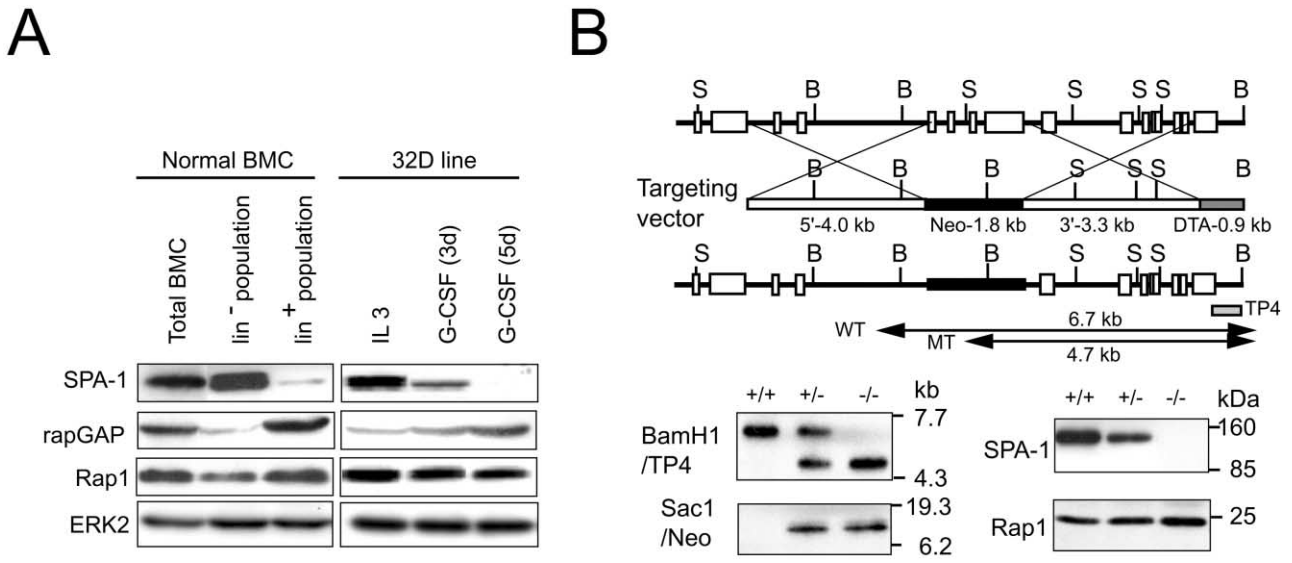

Figure 1. Selective expression of SPA-1 in hematopoietic progenitors and generation of *SPA-1* gene-targeted mice

A: Cell lysates of the total as well as lin$^-$ and lin$^+$ (Gr-1$^+$Mac-1$^+$Ter119$^+$) fractions of BMC from normal B6 mice and 32D promyeloblastic cells before and after the culture with G-CSF (10 ng/ml) were immunoblotted with anti-SPA-1, anti-rapGAP, anti-Rap1, and anti-ERK2 as a loading control. Over 75% of 32D cells were mature granulocytes on day 5 after the culture with G-CSF.

B: Exons 5 to 8 encoding GRD of *SPA-1* gene were replaced by a *pgk-neo* using an indicated targeting vector. BamHI- and SacI-digested genomic DNAs were Southern blotted with *TP4* and *neo* probes, respectively, and spleen cell lysates were immunoblotted with anti-SPA-1 or anti-Rap1.

the progenitors in the BMs of preleukemic SPA-1$^{-/-}$ mice. As shown in Figure 4B, both lin$^-$ c-kit^{high} progenitor cells and lin$^-$ Sca-1$^+$ c-kit^{high} population representing hematopoietic stem cells were significantly increased in the BMC of preleukemic SPA-1$^{-/-}$ mice as compared with the control mice. The mean proportion of lin$^-$ Sca-1$^+$ c-kit^{high} cells (HSC) in SPA-1$^{-/-}$ mice was increased significantly already at 3 months, and was accelerated further at 8 months, while that in the control littermates remained unchanged (Figure 4B). To examine whether the increase reflected hypersensitivity of the progenitors to hematopoietic factors, lin$^-$ c-kit^{high} cells were sorted from the BMC and cultured in the presence of varying concentrations of GM-CSF. As shown in Figure 4C, sensitivity of the isolated progenitors from SPA-1$^{-/-}$ mice to GM-CSF per se was indistinguishable from that of the control mice; this effect was associated also with the comparable differentiation into mature lin$^+$ myeloid cells.

Role of Rap1 signaling in the proliferation of hematopoietic progenitors

The lin$^-$ BMC population pooled from preleukemic SPA-1$^{-/-}$ mice revealed marked accumulation of Rap1GTP as expected, while it was below the detection level in the same fraction of control littermates (Figure 5A). The lin$^-$ BMC from SPA-1$^{-/-}$ mice also showed significantly enhanced ERK activation as compared with the control, while the amount of RasGTP was not increased (Figure 5A). In order to directly investigate the role of Rap1 signaling in the normal progenitors, sorted lin$^-$ cells from the pooled BMC of normal BALB/c mice pretreated with 5-FU were infected in vitro with a recombinant MSCV-IRES/EGFP (MIE) retrovirus containing *RapE63* or *SPA-1* cDNA and cultured on PA6 stroma cells. As shown in Figure 5B, transfection of *RapE63* resulted in the enhanced cellular expansion mostly underneath the stroma cells as compared with that of vector control, while overexpression of SPA-1 caused marked suppression. We also transferred the transfected cells along with normal BMC into the lethally irradiated BALB/c mice. At 3 weeks after the transfer, *RapE63*-transfected progenitors showed enhanced expansion in the BM as compared with the mock-transfected progenitors (Figure 4B), mean GFP$^+$ cell proportions in the original donor population and the recipient BMC being 19% and 32%, respectively, for the former, and 20% and 19% for the latter. FACS analysis revealed that the RapE63-transfected progenitors differentiated comparably to the controls in the BMs and even better in the spleens (Figure 4C). The results suggested that endogenous Rap1 signaling promoted the expansion of hematopoietic progenitors without compromising their differentiation potential.

Crucial role of excess Rap1 signaling in the leukemogenicity of blast cells in vivo

Spleen cells of the SPA-1$^{-/-}$ mice with cp-CML that consisted mostly of mature granulocytes revealed no detectable Rap1GTP like normal spleen cells (Figure 6A), probably reflecting the maturation-related shift of Rap1GAP from SPA-1 to rapGAP (see Figure 1A). In contrast, spleen cells from the mice with CML in blast crisis showed significant Rap1GTP accumulation in proportion to the blast cell contents (Figure 6A); this was compatible with their arrested maturation. They also exhibited marked ERK activation, which was associated with strong Ras activation unlike the preleukemic BM progenitors (Figure 6A). In an attempt to investigate the roles of Rap1 signaling in the leukemic blast cells, we established a cell line (1629B) from a SPA-1$^{-/-}$ mouse with CML in blast crisis. 1629B cells were immature blastic cells (Figure 6B), whose phenotypes were CD45$^+$, Sca-1^{high}, c-kit$^{-/low}$, CD34$^+$, Mac-1^{low}, Gr-1$^-$, Ter $^{-/low}$, B220$^-$, and CD3$^-$. The cells

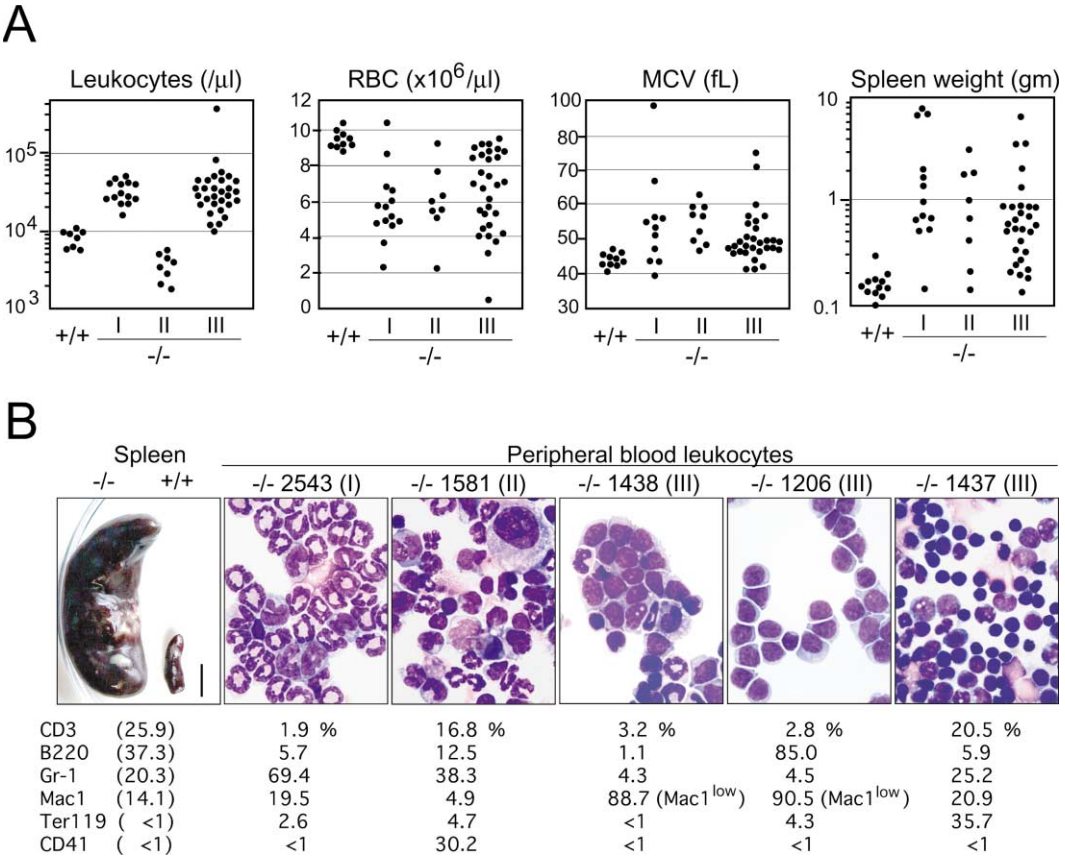

Figure 2. Hematological abnormalities in SPA-1$^{-/-}$ mice

A: Hemograms of 10- to 16-month-old SPA-1$^{-/-}$ and $^{+/+}$ littermates. Abnormalities in the blood leukocytes of SPA-1$^{-/-}$ mice were classified into three groups. I, leukocytosis with few blast cells (14 out of 60 mice); II, pancytopenia (8 out of 60 mice); and III, leukocytosis with varying proportions (14%–85%) of blast cells (35 out of 60 mice).

B: Marked splenomegaly (a bar indicating 10 mm) and abnormal leukocytes in the peripheral blood of SPA-1$^{-/-}$ mice. Heparinized bloods from SPA-1$^{-/-}$ mice were treated with hypotonic buffer to lyse RBC, cyto-spun, and stained with May-Grunwald Giemza solution (original magnification; ×1,000). Aliquots of them were analyzed for the indicated markers using FACScan, the mean percentages of ten control mice being indicated in parenthesis. Representative pictures of groups I to III are shown. Note the dysplastic granulocytes and large megakaryocytic cells in #1581 and abundant erythroblasts in # 1437 SPA-1$^{-/-}$ mice.

could be propagated continuously in the presence of functional stroma cell lines (PA6, Tst-4). They grew underneath the stroma cells like cobblestones (Figure 6B), while they died off within 2 days in the absence of stroma cells (Figure 6C). Neither adherent cell lines incapable of supporting normal hematopoiesis (NIH3T3, endothelial F2) nor any soluble hematopoietic factors could support their survival at all (Figure 6C). Also, culture of them with stoma cells in combination with various hematopoietic factors induced little sign of differentiation (data not shown), suggesting that their differentiation potential was arrested irreversibly. 1629B cells showed large amounts of Rap1GTP as well as strong Ras and ERK activation like the blast cells in vivo (Figure 5D) and caused lethal leukemia in SCID mice, strongly suggesting that they represented the blast cells in the crisis.

In an attempt to abrogate excess Rap1 activation, we infected the 1629B cells with MIE-SPA-1 or an empty MIE retrovirus as a control, sorted twice for GFP^{+} cells up to over 95% purity, and expanded without cloning to avoid clonal variance. As expected, 1629B/SPA-1 cells showed undetectable Rap1GTP, while control cells (1629B/GFP) contained abundant Rap1GTP (Figure 6D), confirming that the accumulation of Rap1GTP was a direct consequence of endogenous SPA-1 deficiency. In contrast, both Ras and ERK activation were not affected in 1629B/SPA-1 cells (Figure 6D), suggesting that the ERK activation was mediated by constitutive Ras activation that was independent of Rap1 signaling in the blast cells. 1629B/SPA-1 cells grew comparably to or even better than 1629B/GFP cells, while they continued to be dependent on the stroma cells for survival. The growth of both cells in the presence of stroma cells was suppressed by a MEK inhibitor, indicating that the constitutive Ras-ERK signaling, but not Rap1 signaling, was responsible for the continuous growth on the stroma cells in vitro (Figure 6D). Nonetheless, when transferred into unirradiated SCID mice, no signs of leukemia developed in any recipients of 1629B/SPA-1 cells, while seven out of eight recipients transferred with the same numbers of 1629B/GFP cells developed blastic leukemia associated with anemia in 7 weeks (Figure 6E). The vast majority of leukocytes in the latter were blast cells of donor origin (H-2^{b}), and all the 7 recipients died by 11 weeks with massive hepatosplenomegaly and blast cell infiltration into the vital organs (Fig-

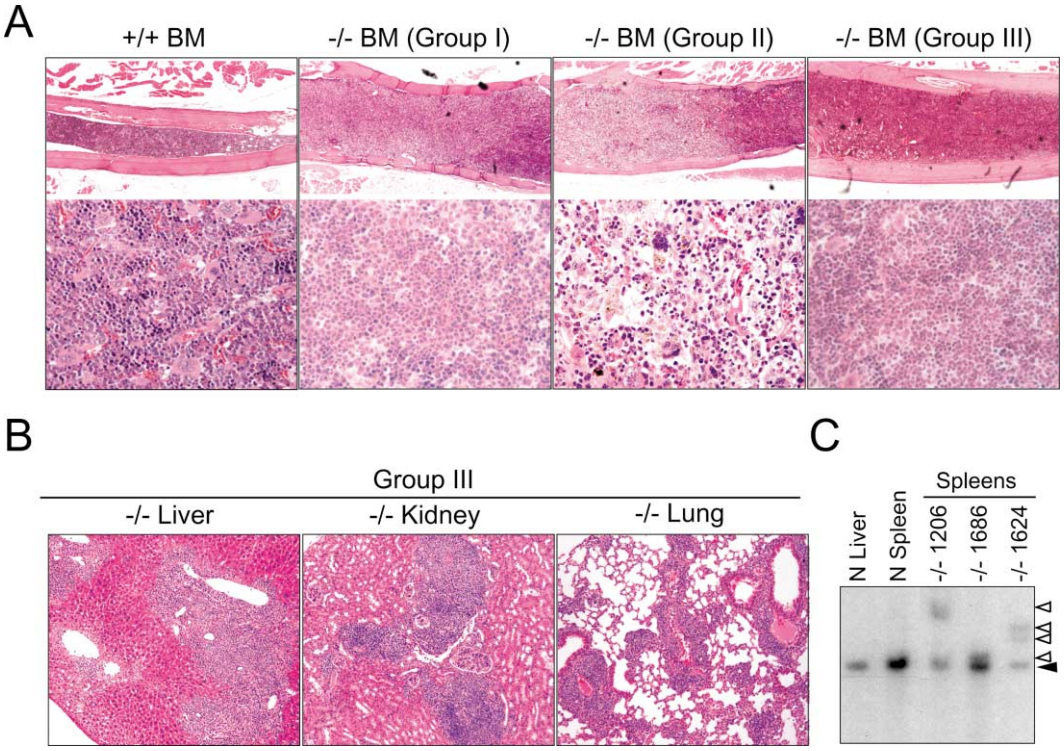

Figure 3. Histology of the BMs and other organs of leukemic SPA-1$^{-/-}$ mice and clonality of blast cells

A: Representative H-E stained pictures of the BMs from diseased SPA-1$^{-/-}$ mice (original magnifications, ×40 and ×400). Note the increase in well-maturated myeloid cells (group I), hypoplastic region (group II), and monotonous blastic cells (group III) all associated with marked reduction of erythroid components.
B: Infiltration of leukemic cells in the vital organs of group III SPA-1$^{-/-}$ mice (H-E stain, original magnification, ×200).
C: Southern blotting analysis of EcoRI and BamHI-digested DNAs from SPA-1$^{-/-}$ spleens containing B220^{+} blast cells as well as control spleen and liver using an immunoglobulin J_H cDNA probe. Solid arrowhead, germline band; open arrowheads, rearranged bands.

ure 6E). Although three out of the eight recipients of 1629B/SPA-1 cells ultimately developed similar leukemia with several weeks delay, the enlarged spleens containing over 90% donor blast cells expressed no *SPA-1* cDNA (Figure 6F), indicating that the leukemia was due to the selective outgrowth of minor 1629B cells without a transfected *SPA-1* cDNA. The results clearly indicated that the persistent Rap1 signaling played a crucial role for the leukemogenicity of blast cells in vivo.

Table 1. Transfer of the myeloproliferative diseases of SPA-1$^{-/-}$ mice into SCID mice

			PB					BMC		
Donor	Cells	Recipient No.	WBC no. (/μl)	Gr-1 (%)	Mac-1 (%)	Ter (%)	SPL weight (mg)	Gr-1 (%)	Mac-1 (%)	Ter (%)
Control	BMC	4	1,480	85.8	5.5		115	46.9	1.3	39.1
			(40)	(0.7)	(3.5)	<1	(52)	(9.9)	(0.5)	(8.7)
#861	SPL	4	17,400	91.2	<1	<1	382	80.4	1.1	11.7
			(2,020)	(3.0)			(44)	(1.0)	(3.9)	(3.0)
		4	Dead before 6 weeks							
			[36,100	65.1	4.1	<1	5,500	65.1	1.1	2.1]
#1438	BMC	2	42,050	9.5	70.2*	8.8	408	39.4	37.4*	8.9
	SPL	2	3,500	33.3	35.4*	10.9	240	64.4	3.7	18.1
	PB	3	42,600	5.4	82.6*	9.9	432	37.9	38.4*	10.7
			(27,900)	(0.8)	(4.2)	(9.2)	(88)	(14.3)	(9.5)	(5.3)
		1	10,000	92.1	<1	8.3	142	85.3	<1	9.1
			[370,000	4.3	88.7*	<1	3,324	53.8	22.5*	11.7]

Cells from the SPA-1$^{-/-}$ mice with cp-CML-like (#861) or AML (#1438), or age-matched control mice were injected i.v. into 2.75 G γ-ray irradiated SCID mice at 5 × 10^6 cells/mouse. Recipient mice were sacrificed at 6 to 9 weeks for histohematological examination. Four out of eight recipients of #861 cells died before 6 weeks escaping the analysis. The vast majority of Mac-1^{+} cells in the #1438 recipients were Mac-1^{low} blasts (*), which heavily infiltrated into vital organs. (): standard errors. []: Data of the original donor mice.

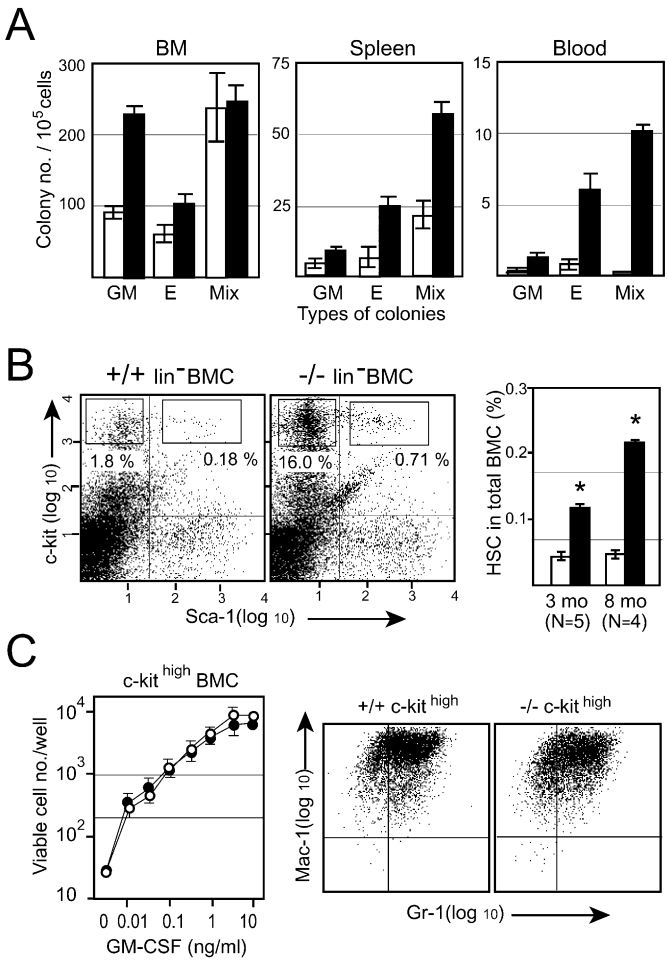

Figure 4. Increase in the hematopoietic progenitors in SPA-1$^{-/-}$ mice

A: CFU-Cs in the BMC, spleen, and PB of a SPA-1$^{-/-}$ mouse with cp-CML (closed bars) and age-matched control littermate (open bars) were quantified using a methylcellulose colony assay. GM: granulocytes/macrophages, E: erythroid, Mix: mixed colonies of GM, E, and megakaryocytic cells. The means and SE of triplicate cultures are indicated. Similar results were obtained in three SPA-1$^{-/-}$ mice.

B: BMC from the 3- and 8-month-old preleukemic SPA-1$^{-/-}$ (closed columns) and control (open columns) littermates were analyzed by three-color staining using FITC-conjugated mixtures of lineage markers (anti-Thy1, anti-B220, anti-Gr-1, anti-Ter-119), PE-conjugated anti-Sca-1, and APC-conjugated anti-c-kit. Representative FACS profiles of the lin$^-$ populations of 8-month-old preleukemic SPA-1$^{-/-}$ and control mice (left two) as well as the mean proportions of lin$^-$ Sca-1^{high} c-kit$^+$ cells (HSC) in the indicated numbers of mice (right) are indicated. * $p < 0.01$.

C: Sorted c-kit $^+$ cells from the BMC of preleukemic 8-months-old SPA-1$^{-/-}$ (●) and control (○) mice were cultured in the presence of varying concentrations of GM-CSF at 200 cells/well. Viable cell numbers were determined on day 6 using a flowcytometry, the means and SE of three mice being indicated (left). Aliquots of the cells were analyzed for Gr-1 and Mac-1 expression (right).

Discussion

SPA-1 gene was isolated originally from a fetal liver-derived immature cell line, whose expression was induced in a proliferative state while repressed in a quiescent state (Hattori et al., 1995), and encodes a specific GAP for Rap1 GTPases (Kurachi et al., 1997). Present results have indicated that SPA-1 is a principal Rap1GAP in the immature hematopoietic progenitors. The vast majority of SPA-1$^{-/-}$ mice developed myeloid leukemia that resembled human CML in cp and in blast crisis from around a year old onward. While 15% of SPA-1$^{+/-}$ mice also developed CML, it remained to be investigated whether the leukemia was associated with mutations or deletion of a wild-type *SPA-1* allele. The mice with cp-CML showed marked increase in the committed progenitors of all the lineages, which was transferable into SCID mice and thus progenitor cell autonomous. Evidence for the increase in pluripotential hematopoietic progenitors was detected in the BM of preleukemic SPA-1$^{-/-}$ mice at as early as 3 months. This increase was accelerated further as mice aged. BM progenitors in the preleukemic SPA-1$^{-/-}$, but not control, mice showed marked accumulation of Rap1GTP as expected. It was indicated that retrovirus-mediated expression of an active mutant of Rap1 (RapE63) significantly enhanced the proliferation of normal hematopoietic progenitors on stroma cells in vitro as well as in the BM of lethally irradiated mice in vivo without compromizing their differentiation. Thus, it was suggested that the persistent Rap1 signaling was responsible for the accelerated hematopoiesis in SPA-1$^{-/-}$ mice. Although there was a significant increase in HSC fraction in the BM of preleukemic SPA-1$^{-/-}$ mice, it remained to be verified carefully whether Rap1 signaling controlled the self-renewal capacity of HSC per se.

The preleukemic progenitors in the BM of SPA-1$^{-/-}$ mice showed enhanced activation of ERK, while Ras activation was comparable to or less than that in the control progenitors. The results implied that Rap1 signaling directly activated ERK, via B-Raf for instance (Mikula et al., 2001), or synergistically potentiated Ras-mediated ERK activation as recently reported in neuronal cells (Bouschet et al., 2003). It was reported that mice deficient for NF-1, a RasGAP, showed enhanced Ras activation and hypersensitivity to GM-CSF leading to the development of CML (Birnbaum et al., 2000; Bollag et al., 1996; Largaespada et al., 1996). Mice lacking JunB expression in myeloid progenitors also showed enhanced proliferation mediated by GM-CSF and developed CML (Passegue et al., 2001). The purified progenitors in SPA-1$^{-/-}$ mice, however, revealed no evidence of hypersensitivity to GM-CSF or other hematopoietic factors, making significant contribution of Rap1 signaling at the downstream of these hematopoietic factors rather unlikely. In addition to the hematopoietic factors, it is shown that adhesive interaction of the progenitors with stroma cells mediated by integrins is essential for the normal hematopoiesis (Arroyo et al., 1999; Miyake et al., 1991), and integrin-mediated adhesion induces ERK activation linking to cell cycle progression (Wary et al., 1996). Considering a pivotal role of Rap1 in integrin activation (Bos et al., 2001; Katagiri et al., 2000), it seems possible that the persistent Rap1 signaling enhances adhesive interaction of the progenitors with stroma cells in the hematopoietic microenvironment. It may be also possible that Rap1 signaling is involved in the proliferation and/or cell survival induced by the stroma-derived soluble factors or cell bound ligands.

Majority of the leukemic SPA-1$^{-/-}$ mice revealed the blast cells of diverse lineages including myeloid, erythroid, and B lineage, which were mono- or oligoclonal. Also, cell transfer studies into SCID mice indicated the evidence for coexistence of cp-CML progenitors and blast cells in a single mouse. Thus, it was suggested strongly that the leukemia represented blast crisis of cp-CML. The blast cells continued to exhibit marked

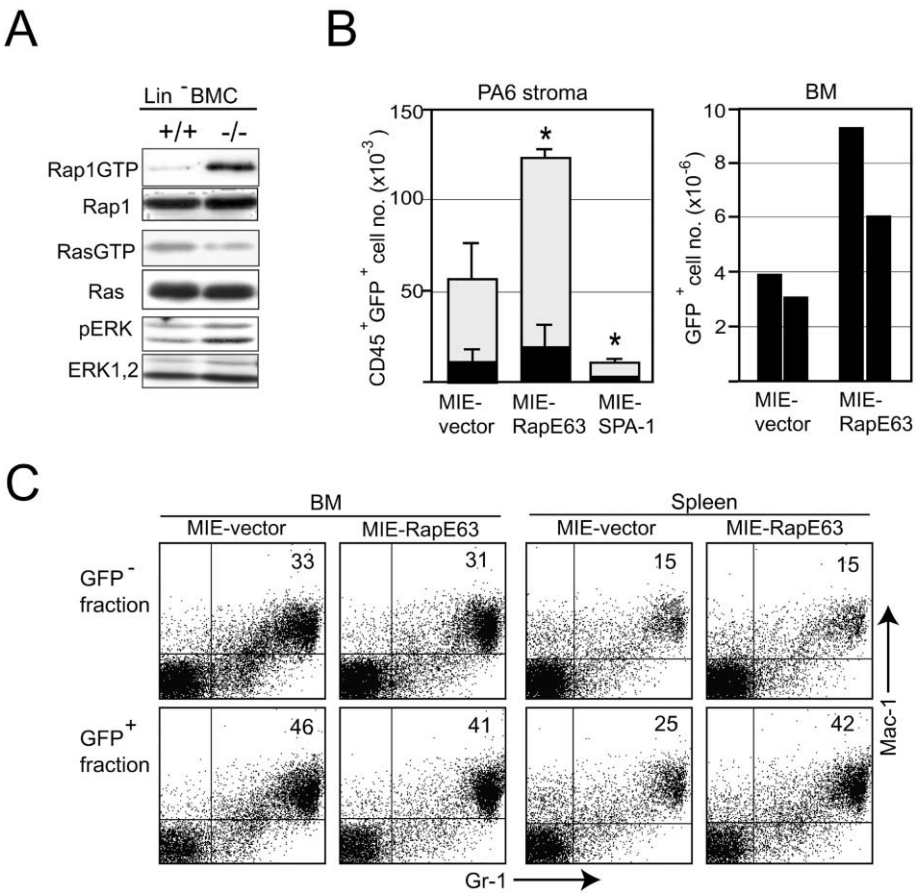

Figure 5. Accumulation of Rap1GTP in the hematopoietic progenitors of preleukemic SPA-1$^{-/-}$ mice and role of Rap1 signaling in normal hematopoiesis

A: Lin$^-$ populations of BMC from preleukemic SPA-1$^{-/-}$, and control mice were lysed, and Rap1GTP/total Rap1, RasGTP/total Ras, and phosphorylated ERK/total ERK were detected.

B: Sorted lin$^-$ BMC from normal BALB/c mice pretreated with 5-FU (150 mg/kg) were infected with MIE retrovirus containing *RapE63* cDNA, *SPA-1* cDNA, or with a vector alone as a control. The infected cells were cultured on PA6 stroma cells at 500 cells/well and the numbers of floating CD45$^+$GFP$^+$ cells (solid columns) and those underneath the stroma cells (shaded columns) were determined on day 10 (left). The means and SE of triplicate cultures are indicated. * $p < 0.05$. They were also transferred into lethally irradiated BALB/c mice (two mice each), and GFP$^+$ cell numbers in the recipient BMs were determined 3 weeks later (right). A solid column represents each recipient.

C: BM and spleen cells from the recipients of MIE/RapE63- or empty MIE-retrovirus-infected progenitors were analyzed for Gr-1 and Mac-1 expression in each of GFP$^-$ and GFP$^+$ fractions.

accumulation of Rap1GTP, consisting with the differentiation arrest at earlier stages before significant rapGAP expression. In contrast to the preleukemic progenitors, the blast cells showed constitutive Ras activation associated with marked ERK activation. To investigate the roles of Rap1 signaling in the blast cells, we established a cell line (1629B) from a SPA-1$^{-/-}$ with CML in crisis, which showed immortalized growth in vitro only in the presence of stroma cells. 1629B cells exhibited both Rap1GTP accumulation and constitutive Ras-ERK activation like the blast cells in vivo and caused lethal leukemia and anemia in SCID mice, indicating that they represented the leukemic blast cells in an original SPA-1$^{-/-}$ mouse. While restoring *SPA-1* gene in 1629B cells completely abrogated Rap1GTP accumulation, as expected, it did not affect the constitutive Ras-ERK activation, indicating that the Ras activation in blast cells was independent of Rap1 signaling. In vitro growth of both 1629B and 1629B/SPA-1 cells in the presence of stroma cells was inhibited completely by a MEK-inhibitor. On the other hand, in spite of the retained Ras-ERK activation after the depletion of stroma cells, they could not survive in the absence of stroma cells (our unpublished observation). Thus, it was suggested strongly that the immortalized growth of 1629B cells was dependent on both constitutive Ras-ERK signaling to promote cell cycle progression and cell survival signal(s) provided by the intimate interaction with stroma cells.

When transferred into SCID mice, 1629B/SPA-1 cells failed to induce leukemia, while control 1629B/GFP cells rapidly caused aggressive leukemia with tissue infiltrations similar to the disease seen in the original SPA-1$^{-/-}$ mice. The results reinforced that the constitutive Ras-ERK activation was necessary but not sufficient for the leukemogenesis in vivo and that additional Rap1 signaling played a crucial role for it. It is indicated that the blast crisis of cp-CML is associated with additional genetic changes (Kabarowski and Witte, 2000; Wolff, 1997). In mouse models, it was shown that the development of AML by leukemogenic fusion genes primarily affecting hematopoietic progenitors such as *PML-RARA*, *AML1-ETO*, and *MLL-AF9* required the cooperating genetic changes (Brown et al., 1997; Corral et al., 1996; Higuchi et al., 2002). For instance, conditional *AML1-ETO* knockin mice that showed enhanced self-renewal potential of HSC developed overt AML only after the additional introduction of potent mutagenesis in vivo (Higuchi et al., 2002). Because the constitutive Ras activation, which was not observed in the preleukemic progenitors, was independent of SPA-1 deficiency per se, it was suggested to reflect the secondary genetic changes. We found no missense mutation in *H-, K-*, and *N-Ras* genes of 1629B cells (M.H. and N.M., unpublished observation), and possible cooperative genes remain to be identified. Considering the critical role of stroma cells for the immortalization of 1629B cells in vitro, we speculate that Rap1 signaling may be required for the homing and invasion of blast cells into the appropriate tissue environment and/or successful interaction with proper stroma cells there to develop leukemia (Shimonaka et al., 2003; Uemura and

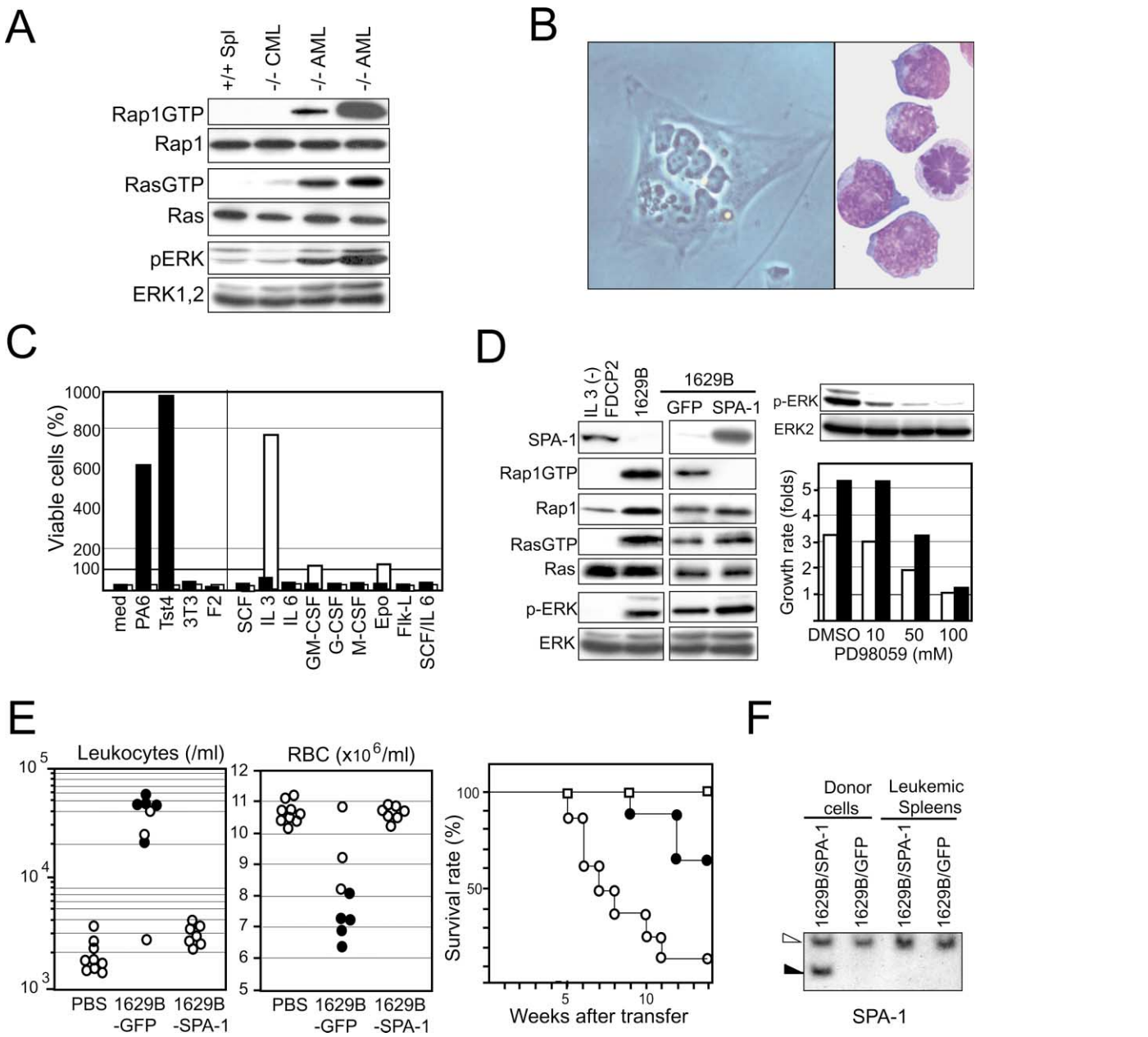

Figure 6. Accumulation of Rap1GTP in the blast cells in vivo and suppression of the leukemogenicity of a blast cell line by restoring *SPA-1* gene

A: Spleen cells from control mouse, SPA-1$^{-/-}$ mouse with cp-CML (over 75% granulocytes) and those in blast crisis (the third lane, 20% blast cells,and the forth lane, 80% blast cells) were lysed and examined for the activation of Rap1, Ras, and ERK.

B: Phase-contrast picture of 1629B cells growing underneath a stroma cell and May-Giemza stained picture (original magnifications, ×200 and ×1000, respectively).

C: 1629B (solid columns) and IL-3-dependent FDC-P2 cells (open columns) were cultured in the absence or presence of hematopoiesis-supporting stroma cells (PA6, Tst-4), other adherent cells (NIH3T3, F2 endothellial line), or various hematopoietic growth factors for 4 and 2 days, respectively, and the % viable cell numbers of inputs were determined.

D: Parental 1629B cells, 1629B cells infected with empty MIE retrovirus (1629B/GFP), and those infected with MIE/SPA-1 (1629B/SPA-1) were lysed and immunoblotted with indicated antibodies. A faint signal of SPA-1 in 1629B/GFP cells was due to the minor contaminated PA6 stroma cells. IL-3-starved FDC-P2 cells served as a negative control for Rap1, Ras, and ERK activation (left). 1629B/GFP (open columns) and 1629B/SPA-1 (solid columns) were plated on the monolayers of PA6 stroma cells, PD98059 was added at varying concentrations on day 1, and viable cell numbers were determined on day 3. The mean growth rates (fold increase in the cell numbers) of duplicate cultures as well as the ERK activation are indicated (right). Results of the latter were identical in the two cell lines, only that of 1629B/SPA-1 being indicated. Note that PD98059 at 100 μM induced complete inhibition of cell growth but no cell death, unlike the depletion of stroma cells that resulted in the entire cell death.

E: Unirradiated SCID mice were injected i.v. with 1629B/GFP, 1629B/SPA-1, or PBS as a control at one million cells/mouse, and the numbers of leukocyte and RBC in the PB were counted at 8 weeks (left two). Solid circles indicate the recipients that died before 8 weeks. Survival rates of above groups of mice (□, PBS, 9 mice; ○, 1629B/GFP, 8 mice; ●, 1629B/SPA-1, 8 mice) were monitored for over 3 months (right).

F: Presence of transfected *SPA-1* cDNA was examined by Southern blotting in the enlarged spleens of leukemic mice injected with 1629B/GFP (at 8 weeks) or 1629B/SPA-1 cells (at 12 weeks), both of which contained over 95% donor-derived (H-2K^{b}) blast cells. Inoculated 1629B/SPA-1 and 1629B/GFP cells served as controls. DNAs were digested with BamHI and probed with a *SPA-1* cDNA. An open arrowhead indicates a genomic (mutated) *SPA-1* gene (4.7 kb) and a solid arrowhead a transfected *SPA-1* cDNA (3.2 kb).

Griffin, 1999). It also remains to be seen whether persistent Rap1 signaling predisposes the secondary genetic changes per se in blast crisis.

Long latency was a characteristic feature of the leukemia in SPA-1$^{-/-}$ mice, even though significant expansion of the hematopoietic progenitors within BM was evident as early as 3 months. Immune functions of the T cells were normal or rather enhanced in these young SPA-1$^{-/-}$ mice. After around half a year, however, SPA-1$^{-/-}$ mice were found to develop progressive T cell immunodeficiency preceding the leukemia development, which was associated with the increasing accumulation of Rap1GTP in the memory phenotype T cells (Ishida et al., 2003). Unlike in the hematopoietic progenitors, excess Rap1GTP in the antigen-primed T cells interfered with the Ras-mediated ERK activation and proliferation via antigen receptor stimulation leading to anergic state, which might be related to rare T cell leukemia in SPA-1$^{-/-}$ mice. Opposite functional effects on T and myeloid cells were reported also in the mice expressing *AML1-ETO* oncogene (Higuchi et al., 2002). There are indications that immune system plays a significant role in the control of myeloproliferative diseases in human and mice (Faderl et al., 1999; Kolb et al., 1995; Pear et al., 1998), and it is tempting to speculate that the long latency of leukemia might partly reflect the characteristic age-dependent alteration in the T cell functions during preleukemic stages.

A minor group of SPA-1$^{-/-}$ mice developed pancytopenia associated with dysplasic leukocytes, reminiscent of human MDS. MDS is also a hematopoietic stem cell disorder, sharing several features with CML, including the occurrence in elderly population and high risk of progression to AML (Kouides and Bennett, 1996; Heaney and Golde, 1999). It remained to be examined carefully whether part of blastic leukemia in SPA-1$^{-/-}$ mice was preceded by the MDS-like condition. Although factors affecting the variable disease types in SPA-1$^{-/-}$ mice remained to be determined, possible factors might include the genetic backgrounds, either 129 or C57Bl/6, and the altered T cell functions that could affect normal hematopoiesis. Development of severe often life-threatening macrocytic anemia was observed in the vast majority of SPA-1$^{-/-}$ mice, and our unpublished results indicated that in vitro erythroid cell differentiation from SPA-1$^{-/-}$ ES cells was impaired at the terminal stages, which could be compensated for by erythropoietin (T. Nakano and N.M., unpublished observations). Thus, the anemia could be due in part to the intrinsic effects of SPA-1 deficiency on erythropoiesis in addition to the ineffective erythropoiesis secondary to the myeloid leukemia. Human *SPA-1* gene is mapped at chromosome 11q13 (Wada et al., 1997), one of the cytogenetically promiscuous sites in human hematological malignancy (Wong, 1999), and possible involvement of *SPA-1* gene in human myeloproliferative disorders needs to be investigated.

Experimental procedures

Generation of SPA-1-deficient mice

E14 ES cells were transfected with a linearized *SPA-1* gene-targeting vector, in which a region covering exons 5 to 8 encoding GRD was replaced by a 1.8 kb fragment containing a *pgk-neo* cassette, by electroporation and selected in the medium containing G418 (125 μg/ml) followed by the screening with Southern blot analysis using TP4 and neo probes (Figure 1B). Correctly targeted ES clones were microinjected into C57BL/6 (B6) blastocysts, and the resulting chimaeras were mated with B6 mice. Heterozygous offspring were intercrossed to produce homozygous mutant mice, and those with mixed genetic backgrounds were used in the present study. SCID mice were obtained from CLEA Japan, Inc.

Cells and cultures

Stroma (PA6, Tst-4) and other adherent cell lines (NIH3T3, F2) as well as myeloid (32D, FDC-P2) cell lines were maintained in RPMI1640 medium supplemented with 10% FCS with additional IL-3 for the latter. To induce 32D cell differentiation, the cells were washed and cultured in the presence of 10 ng/ml G-CSF. 1629B cell line was established from the Dexter-type culture of BMC from a SPA-1$^{-/-}$ mouse with CML in blast crisis. After 8 weeks of the primary culture, cobblestone-like cell clusters that developed underneath the stroma cells were collected and propagated on a monolayer of PA6 stroma cells in the modified DMEM with 10% FCS, 10% NCTC109 medium, 10^{-5}M 2-mercaptoethanol, 100 U/ml insulin, 1 mM sodium pyruvate, 1 mM oxalacetic acid, 0.1 mM NEAA, and 10 mM HEPES (Dupuy et al., 2001). To separate 1629B and stroma cells, the trypsinized cell suspension was incubated with rat anti-VCMA-1 antibody and then with anti-rat-Ig-conjugated Dinabeads (Dynal ASA) followed by magnetic beads separation.

Flow cytometry

Two- or three-color flowcytometric analysis was performed with various combinations of lineage-specific monoclonal antibodies using FACScan (Beckton Dickinson). Antibodies included anti-Thy1, anti-CD3, anti-B220, anti-Gr-1, anti-Mac-1, anti-CD41, anti-Ter119, anti-Sca-1, and anti-c-kit (Pharmingen). To separate lin$^-$ and lin$^+$ populations of BMC for biochemical analysis, the BMC suspensions were incubated with the cocktail of PE-conjugated antibodies (anti-Thy1, anti-B220, anti-Gr-1, and anti-Ter119) followed by anti-PE antibody-conjugated magnetic beads and separated using Auto-Max beads columns (Meltenyl Biotech). A lin$^-$ Sca-1^{high} c-kit$^+$ cell fraction was obtained by cell sorting using a FACS Vantage (Beckton Dickinson).

Immunoblotting, detection of Rap1GTP, and Southern blotting

For immunoblotting, cells were lysed with lysis buffer (0.5% Triton-X100, 150 mM NaCl, 50 mM Tris-HCl [pH 7.6], protease and phosphatase inhibitors) with additional 0.1% SDS for BMC and blotted with anti-SPA-1 (Tsukamoto et al., 1999), anti-rapGAP, anti-ERK-1, 2, anti-phosphorylated ERK, anti-Ras, or anti-Rap1 (Santa Cruz Biotechnology) followed by peroxidase-labeled second antibodies. To detect Rap1GTP and RasGTP, cell lysate (0.5 to 1 mg proteins) was precipitated with GST fusion proteins of RalGDS-RBD and c-Raf-1-RBD coupled with glutathione-Sepharose beads for an hour in ice, washed, eluted with SDS sample buffer, and immunoblotted with anti-Rap1 and anti-Ras antibodies, respectively. DNAs of liver and spleens were digested with EcoRI and BamHI, and Southern blotting was done using an immunoglobulin J_H gene cDNA as a probe.

Histohematologic procedures

Blood sampling was made routinely from the retro-orbital plexus, and the numbers of leukocytes and RBC as well as the mean corpuscular volume (MCV) were counted using an automated cell counter (Nihon Kohden, Tokyo). Cytospin preparations of RBC-lysed blood were stained with May-Grunwald Giemza solution, and organs were fixed in 10% formalin and stained with hematoxylin-eosin solution. CFU-C assay was performed as described before (Nishi et al., 1990). Briefly, cell suspensions from BM, spleen, and PB (4×10^5 or 5×10^5 cells/35 mm dish) were cultured in the serum-free methylcellulose medium (α-MEM, 0.9% methylcellulose, 1% deionized BSA, 0.05 mM 2-mercaptoethanol) supplemented with SCF (100 ng/ml), IL-6 (100 ng/ml), IL-3 (20 ng/ml), erythropoietin (2 U/ml), G-CSF (10 ng/ml), and thrombopoietin (10 ng/ml). Numbers of various types of CFU-C were scored at day 10.

Retrovirus infection

Lin$^-$ BMC were sorted using a cell sorter from normal BALB/c mice injected with 5-FU (150 mg/kg) 5 days before, cultured in the complete RPMI containing SCF (50 ng/ml), Flk-L (10 ng/ml), IL-6 (10 ng/ml), and IL-11 (10 ng/ml) for 1 day, infected with recombinant MIE retrovirus (Hawley et al., 1994) containing *RapE63* or *SPA-1* cDNA. Infection efficiency was 20% to 25%. 1629B cells depleted of stroma cells were infected with recombinant MIE-SPA-1 or empty MIE retrovirus as a control for 3 hr, cultured on the PA6 stroma cells for a week, and GFP$^+$ cells were sorted twice using a cell sorter.

Transfected *SPA-1* cDNA was detected by Southern blotting of the BamHI-digested DNA.

Cell transfers

Cells from PB, spleens, and BMs of SPA-1$^{-/-}$ mice or control littermates were washed in PBS and injected intravenously to the 2.75 Gy γ-ray-irradiated SCID mice at 5–10 × 10^6 cells/mouse. 1629B cells were transferred intravenously into unirradiated SCID mice at 10^6 cells/mouse. BM progenitors of normal BALB/c mice infected with MIE-retrovirus were transferred intravenously into 9 Gy γ-ray-irradiated BALB/c mice at 10^5 cells/mouse along with normal BMC (10^5 cells/mouse).

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