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Spred-1 Negatively Regulates Interleukin-3-mediated ERK/Mitogen-activated Protein (MAP) Kinase Activation in Hematopoietic Cells*

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Sprouty/Spred family proteins have been identified as negative regulators of growth factor-induced ERK/mitogen-activated protein (MAP) kinase activation. However, it has not been clarified whether these proteins regulate cytokine-induced ERK activity. We found that Spred-1 is highly expressed in interleukin-3 (IL-3)-dependent hematopoietic cell lines and bone marrow-derived mast cells. To investigate the roles of Spred-1 in hematopoiesis, we expressed wild-type Spred-1 and a dominant negative form of Spred-1, ΔC-Spred, in IL-3- and stem cell factor (SCF)-dependent cell lines as well as hematopoietic progenitor cells from mouse bone marrow by retrovirus gene transfer. In IL-3-dependent Ba/F3 cells expressing c-kit, forced expression of Spred-1 resulted in a reduced proliferation rate and ERK activation in response to not only SCF but also IL-3. In contrast, ΔC-Spred augmented IL-3-induced cell proliferation and ERK activation. Wild-type Spred-1 inhibited colony formation of bone marrow cells in the presence of cytokines, whereas ΔC-Spred-1 expression enhanced colony formation. Augmentation of ERK activation and proliferation in response to IL-3 was also observed in Spred-1-deficient bone marrow-derived mast cells. These data suggest that Spred-1 negatively regulates hematopoiesis by suppressing not only SCF-induced but also IL-3-induced ERK activation.

Receptor tyrosine kinases, such as stem cell factor (SCF)[1] receptor (c-kit), as well as cytokine receptors including interleukin (IL)-3 or erythropoietin (EPO) receptor activate the extracellular signal-regulated kinase (ERK) cascade. ERK activation is initiated by binding of Grb2 to the phosphorylated tyrosine residues of the receptor or phosphorylated adaptor molecules such as Shc, FRS-2, IRS-1/2, SHP-2, and Gab-1. The complex of Grb2 and SOS (son of sevenless) activates Ras by GTP loading. Ras-GTP recruits Raf1 to the plasma membrane (1, 2), which is then phosphorylated and activated by several, not well defined, kinases with complex regulatory mechanisms (3–5). Activated Raf then phosphorylates and activates the dual-specific kinase MEK, which phosphorylates and activates ERKs. In addition, the Ras-independent Raf1-ERK activation mechanism has been recently demonstrated, and members of the protein kinase C family of serine/threonine kinases have been implicated as potential activators of Raf (6).

Mitogen-activated protein (MAP) kinases including ERKs play important roles in hematopoiesis. Most hematopoietic cytokines (hematopoietins) activate the JAK-STAT and Ras-ERK pathways, both being required for a satisfactory level of proliferation and differentiation of hematopoietic cells. For example, STAT5 activation is not sufficient for EPO-dependent growth of CTLL2 cells expressing EPO receptor, but additional activation of MAP kinases can support their cellular proliferation in response to EPO (7). MAP kinases have also been shown to play a critical role in megakaryopoiesis by c-*mpl* (8). However, little is known about how MAP kinase is regulated in hematopoietic cells. One of important regulators of MAP kinase is the MAP kinase-specific dual specificity protein phosphatase (MKP), which targets both phosphothreonine and phosphotyrosine on most activated MAP kinases (9). However, the physiological relevance of MKPs in hematopoiesis has not been clarified.

Recently, we cloned a family of novel membrane-bound molecules, Spreds, that are related to Sproutys (10). *Drosophila* Sprouty was identified as a negative regulator for several growth factor-induced ERK activation, including FGF and EGF (11, 12). Four Sprouty homologues are found in mammals. Vertebrate Sproutys have also been implicated in the negative feedback regulation of FGF signaling in embryogenesis (11) and angiogenesis (12), although their inhibitory mechanisms are still controversial (13–16). We have identified three members of the Spred family of proteins in mammals (17) that have a Sprouty-related C-terminal cysteine-rich (SPR) domain in addition to the N-terminal Ena/VASP homology (EVH) 1 domain. The SPR domain of Sprouty, and probably Spred, is palmitoylated; thereby, Sprouty and Spred localize in membrane fraction. Like Sprouty, Spred-1, Spred-2, and Spred-3 also down-regulate growth factor-mediated Ras/ERK signaling.

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[1] The abbreviations used are: SCF, stem cell factor; IL, interleukin; EPO, erythropoietin; AGM, aorta-gonad-mesonephros; BMMC, bone marrow-derived mast cells; ERK, extracellular signal-regulated kinase; JAK, Janus kinase ; JNK, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MKP, MAPK phosphatase-1; STAT, signal transducers and activators of transcription; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; VASP, vasodilator-stimulated phosphoprotein; EVH, Ena/VASP homology; BM, bone marrow; BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte-macrophage; CFU-GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte; G-CSF, granulocyte colony-stimulating factor; GFP, green fluorescent protein; EGFP, enhanced GFP; WT, wild type; IRES, internal ribosome entry site; KBD, c-kit-binding domain; SPR, Sprouty-related C-terminal cysteine-rich.

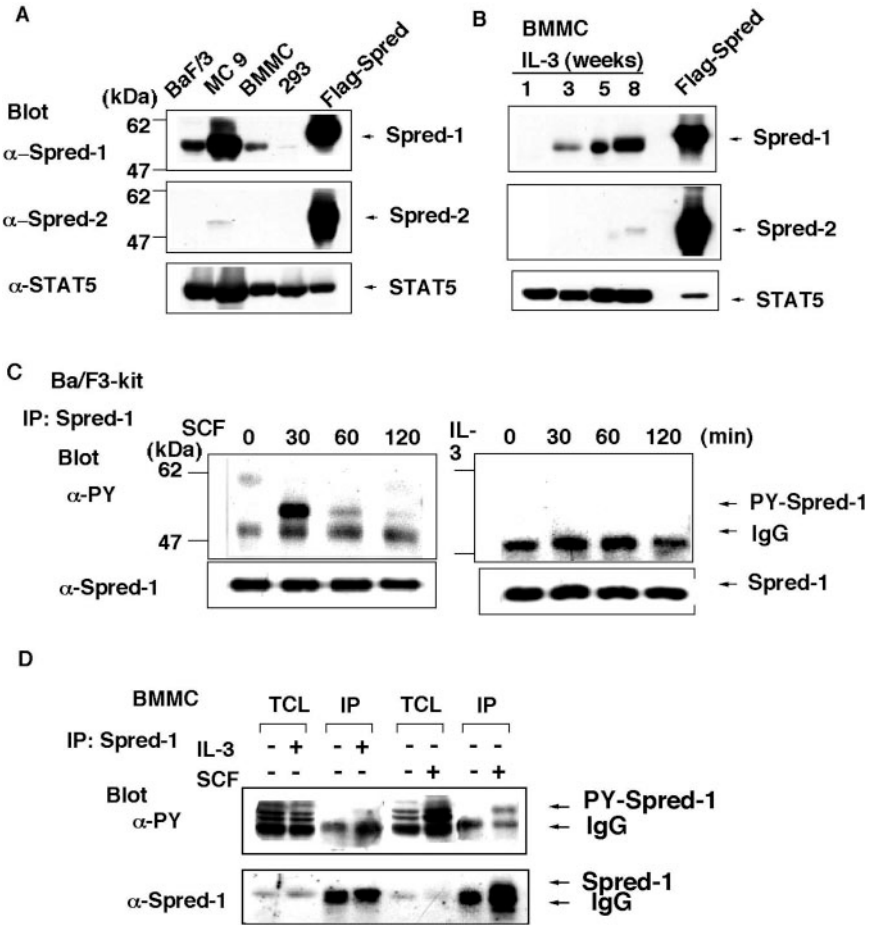

FIG. 1. **Expression of Spred-1 and Spred-2 in hematopoietic cells and tyrosine phosphorylation of endogenous Spred-1 in response to SCF.** *A*, cell extracts of Ba/F3 cells, MC9 cells, BM cells cultured in IL-3 for 3 weeks, and 293 cells transfected with or without FLAG-tagged Spred-1 or Spred-2 were immunoblotted with the indicated antibodies. *B*, expression of Spred-1 in BMMC. Mouse BM cells were cultured in RPMI 1640 containing 10% fetal calf serum and 5 ng/ml IL-3 for the indicated periods. Cell extracts at indicated times were immunoblotted with the indicated antibodies. FLAG-tagged Spred-1 and Spred-2 expressed in 293 cells are used as positive controls. *C* and *D*, Ba/F3-kit cells (*C*) and BMMCs (*D*) were stimulated with 50 ng/ml IL-3 or SCF for the indicated periods. Cell extracts were immunoprecipitated (*IP*) with anti-Spred-1 antibody and were blotted with the indicated antibodies. *PY*, phosphotyrosine; *TCL*, total cell lysate.

We have shown that Spred interacts with Ras and inhibits Raf kinase activation without reducing Ras activation, and interestingly, it stabilizes EGF-induced Raf-1 translocation from cytosol to plasma membrane (10). As Spred inhibits active-Ras-induced ERK activation, Spred might modulate the unidentified activation steps of Raf by a novel mechanism. It was also revealed that the C-terminal deletion mutant of Spred-1 functions as a dominant negative form against endogenous Spred-1 and augments serum- and NGF-induced ERK activation. Spred/Sprouty family proteins have emerged as negative regulators of the ERK pathway; however, details of the physiological functions of each member remain to be investigated.

We have shown that Spred-2 was expressed in the aorta-gonad-mesonephros (AGM) region and negatively regulates AGM hematopoiesis (18). In contrast, we found that expression of Spred-1, but not Spred-2, is high in mature hematopoietic cells. In this study, we demonstrated that Spred-1 is a negative regulator of ERK activation in response to not only SCF but also to cytokines such as IL-3. Spred-1 is also demonstrated to be a physiological negative regulator of bone marrow hematopoiesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Mast Cell Cultures—HEK293 cells and the retrovirus packaging cell line, Plat-E, were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The murine pro-B cell-derived cell line, Ba/F3, was cultured in RPMI medium containing 10% fetal bovine serum and 10% conditioned medium from WEHI-3B cells as a source of IL-3. Ba/F3 cells expressing c-kit (Ba/F3-kit) were established as described (19). A murine mast cell-derived cell line, MC9, was cultured in RPMI medium containing 5% fetal bovine serum, 50 μM 2-mercaptoethanol, and 5 ng/ml IL-3.

Bone marrow-derived mast cells (BMMCs) were obtained from a culture of bone marrow cells in RPMI 1640 supplemented with 5 ng/ml murine IL-3, 10% fetal calf serum, non-essential amino acids (Invitrogen), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-mercaptoethanol.

Spred-1 Knockout Mice—A targeting vector construction, targeting of embryonic stem cells, generation of chimeric mice, and detailed analysis of the knockout mice will be published elsewhere.

Plasmids, Transfection, and Infection—Wild-type Spred-1 and C-terminal-truncated Spred-1 (ΔC-Spred-1) were subcloned into pCDNA3 for the 6× Myc epitope tag or pMY-IRES-EGFP for retrovirus infection as described (20). Various mutants of Spred-1 (ΔC, ΔN1, ΔKBD, ΔIR, R247A) in pcDNA3 were described previously (10). pCDNA3 vectors were transfected into 293 cells on 6-well dishes by the calcium phosphate method. pMX-IRES-EGFP vectors were transfected into a Plat-E packaging cell line using FuGENE 6 (Roche Applied Science) to obtain the viruses (21). Ba/F3-kit cells (2×10^5 cells) were infected with viruses on a RetroNectin (Takara Bio inc.)-coated plate for 24 h in the presence of 5 ng/ml IL-3. Cells were washed three times with phosphate-buffered saline, resuspended in RPMI 1640–10% fetal bovine serum containing 5 ng/ml IL-3 or 100 ng/ml SCF, and incubated for the indicated times. Then, cells (1×10^4) were analyzed for enhanced GFP (EGFP) fluorescence on a Coulter EPICS-XL flow cytometer. GFP-ERK was a gift from Dr. Y. Goto (Tokyo University).

Cell Proliferation Assay—Cell proliferation was assayed using a cell counting kit (Dojindo Laboratories). Briefly, 5×10^3 cells were plated in 96-well plates in RPMI 1640 medium with various concentrations of SCF or IL-3 and cultured for 72 h. After adding WST-1 and 1-methoxy phenazine methosulfate, the optical density for 450 nm was measured.

Immunochemical Analysis—Immunoprecipitation and immunoblotting were performed using anti-Myc, anti-c-kit, anti-STAT5, anti-ERK2, anti-JNK, anti-G-CSF receptor (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-phospho STAT5, anti-phospho ERK2, anti-Akt (Cell signaling), anti-pY (Transduction laboratories), anti-phospho JNK (Calbiochem), and anti-phospho Akt (Biosourse International) as described (20, 22). Anti-Spred-1 and -2 antibodies were prepared by immunizing rabbits (10).

In Vitro Colony Assay—Single cell suspensions were isolated from the bone marrow of C57BL/6 mice, and red blood cells were lysed using

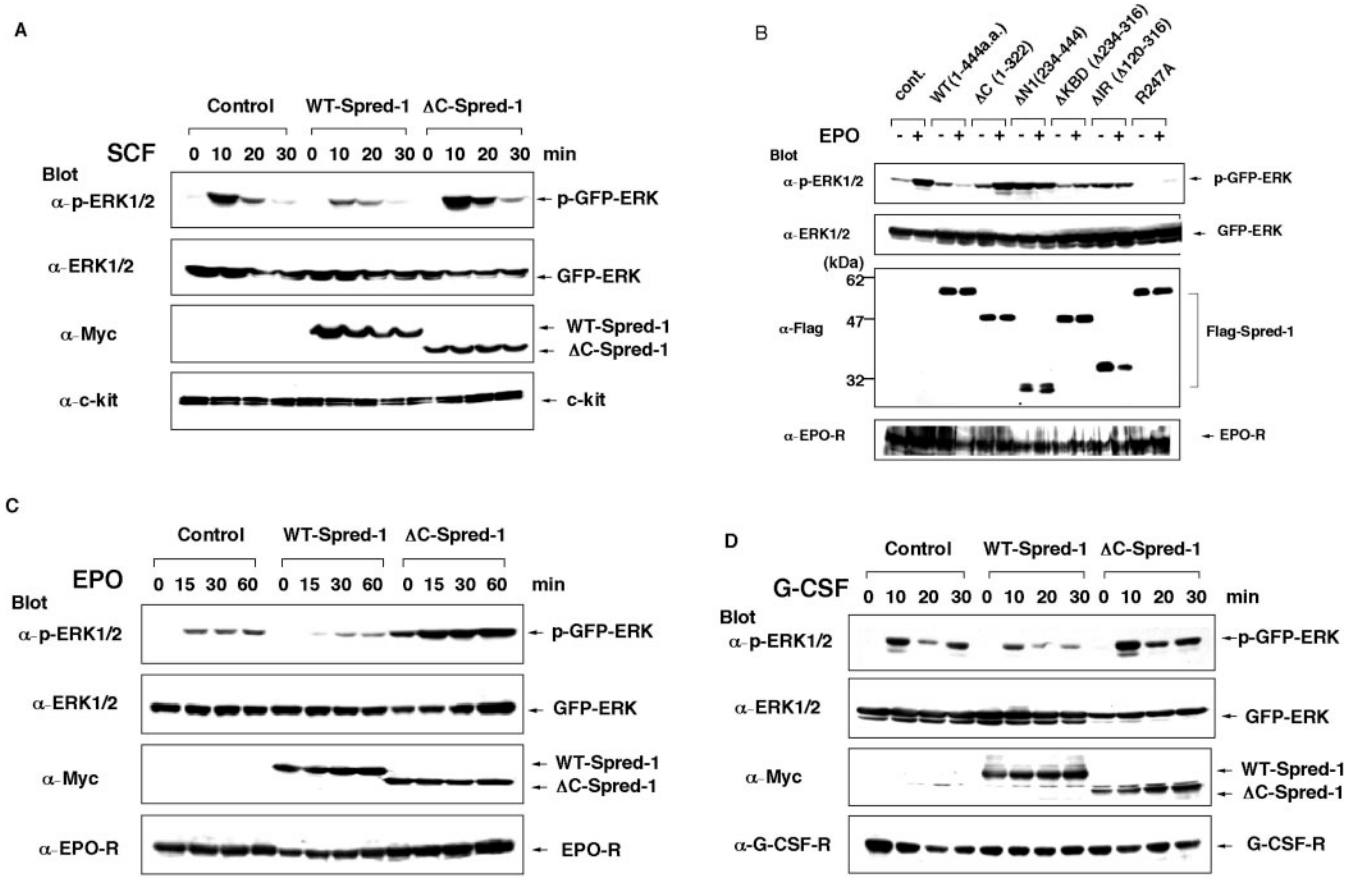

FIG. 2. **Effect of wild-type and various mutants of Spred-1 on SCF, EPO, or G-CSF-induced ERK activation.** 293 cells transfected with wild-type or ΔC-Spred-1 with GFP-ERK and c-kit (*A*) or EPO receptor (*C*) or G-CSF receptor (*D*) were stimulated with 50 ng/ml SCF (*A*) or 20 units/ml EPO (*C*) or 50 ng/ml G-CSF (*D*) for the indicated periods. In *B*, 293 cells transfected with wild-type (WT) or various mutants of Spred-1 (ΔC, ΔN1, ΔKBD, ΔIR, R247A) with GFP-ERK and EPO receptor were stimulated with 20 units of EPO for 10 min. Cell extracts were immunoblotted with the indicated antibodies. *p-ERK*, phosphorylated ERK; *p-GFP-ERK*, phosphorylated GFP-ERK; *EPO-R*, EPO receptor.

NH_4Cl. These cells were infected with the retroviruses (control, wild-type Spred-1, ΔC-Spred-1) on a RetroNectin (Takara)-coated plate for 48 h. GFP-positive bone marrow cells were sorted by EPICS ALTRA (Beckman Coulter), and 3×10^3 GFP-positive cells were plated in methylcellulose (Methocult3434, Stem Cell Technologies Inc.) containing 5 ng/ml IL-3 and 50 ng/ml SCF, IL-6, and 3 units of EPO. On day 14, colonies were enumerated and stained for differential counting (23). The numbers of colonies for colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E), and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were counted microscopically.

RESULTS

Expression Spred-1 in Hemopoietic Cells—To examine the expression of Spred-1 or Spred-2 in hematopoietic cells, we performed Western blotting analysis. As shown in Fig. 1*A*, Spred-1 was detected in two IL-3-dependent cell lines, Ba/F3 and MC9. Spred-1 was also detected in mature BMMCs. We could not detect Spred-1 in non-hematopoietic 293 cells (Fig. 1*A*). Spred-2 expression was not detected by Western blotting, suggesting that the expression levels of Spred-2 in Ba/F3 cells are not as high as Spred-1 (Fig. 1*A*). Spred-1 expression levels increased during culture of bone marrow (BM) cells in the presence of IL-3. Spred-1 was not detected in freshly isolated BM cells, whereas maximum expression was observed after 5–8 weeks of culture in IL-3, in which most of the cells become BMMCs (Fig. 1*B*). We could not detect Spred-1 protein in neutrophils (data not shown). These data suggest that Spred-1 is highly expressed in a particular subset of hematopoietic cells or that Spred-1 expression was induced by IL-3.

We have shown that exogenous Spred-1 was tyrosine-phosphorylated in response to SCF in 293 cells expressing c-kit. To examine the phosphorylation of endogenous Spred-1, Ba/F3 cells expressing c-kit (Ba/F3-kit) were stimulated with SCF. As shown in Fig. 1*C*, endogenous Spred-1 was phosphorylated in response to SCF in Ba/F3-kit cells. We also observed SCF-induced tyrosine phosphorylation in BMMCs (Fig. 1*D*). On the other hand, we could not detect IL-3-induced phosphorylation of Spred-1 in Ba/F3-kit cells and BMMCs (Fig. 1, *C* and *D*). These data indicate that endogenous Spred-1 is tyrosine-phosphorylated by hematopoietic growth factor stimulation.

Suppression of Cytokine-induced ERK Activation by Spred-1—We have shown that wild-type Spred-1 suppressed EGF- and FGF-induced ERK activation and that the C-terminal deletion mutant of Spred-1 (ΔC-Spred-1) functions as a dominant negative form against NGF and serum stimulation (10). We first confirmed that overexpression of wild-type (WT) Spred-1 suppressed SCF-induced ERK activation as compared with the control, whereas ΔC-Spred-1 augmented ERK activation in 293 cells (Fig. 2*A*). Then, we investigated the effect of Spred-1 on cytokine receptor signaling, which utilizes JAKs. First, using various Spred-1 mutants, we examined which part of the protein is necessary for suppressing EPO-induced ERK activation. As shown in Fig. 2*B*, WT-Spred-1 suppressed EPO-induced ERK activation. Similarly, a mutant lacking the internal region between the EVH domain and KBD domain (ΔIR) and mutants of the kit binding domain (ΔKBD and R247A) suppressed EPO-induced ERK activation, indicating that the internal region and KBD are not essential for suppression of ERK. These are similar to our previous results obtained for EGF-induced ERK activation (10). However, ΔIR and ΔKBD seemed to have a weaker effect than R247A and WT-Spred-1.

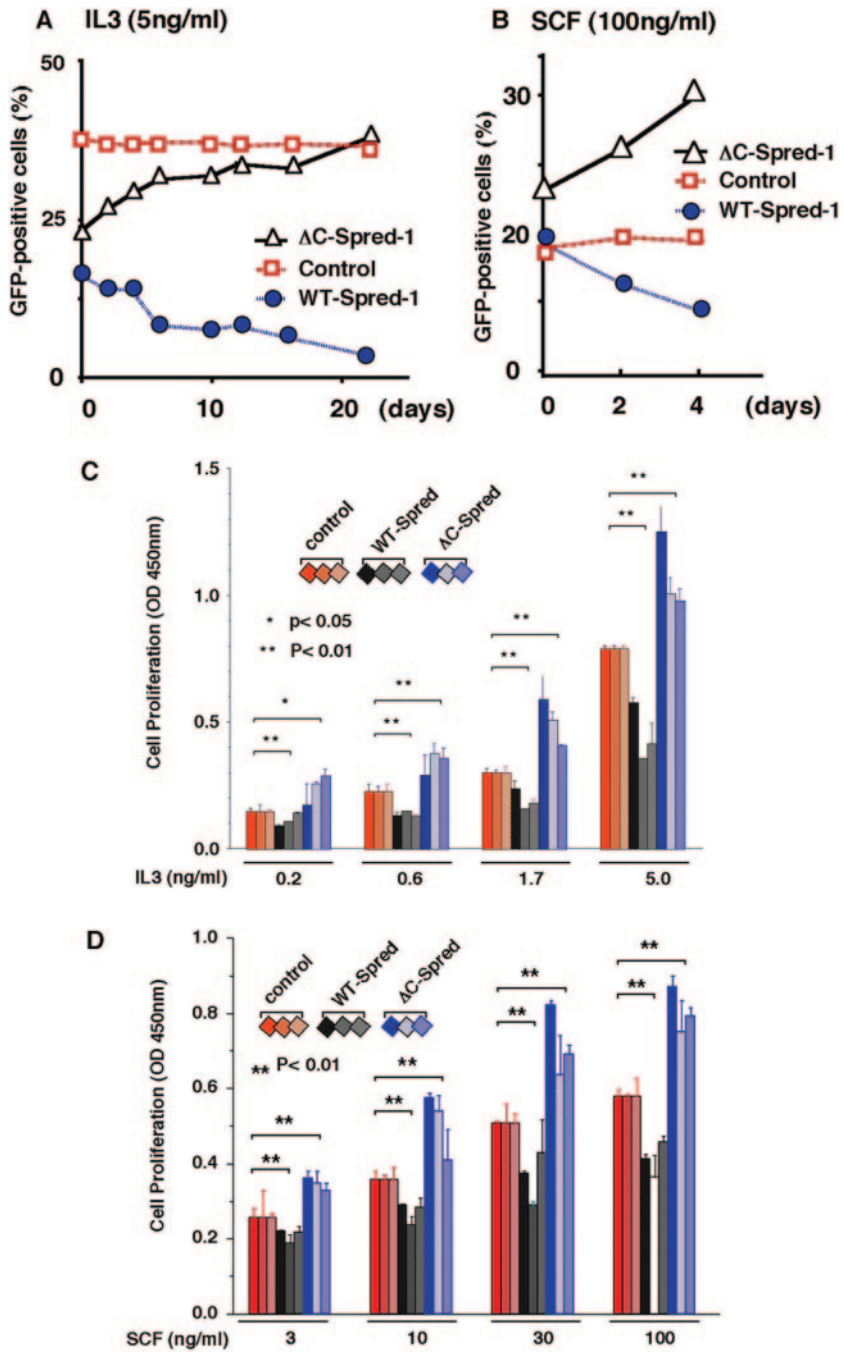

FIG. 3. **Effect of wild-type and ΔC-Spred-1 on proliferation of Ba/F3-kit cells.** *A* and *B*, retroviruses carrying FLAG-tagged wild-type and ΔC-Spred-1 cDNAs in pMY-IRES-GFP vector were produced by transfection of the Plat-E packaging cell line. Ba/F3-kit cells were infected with viruses for 48 h in the presence of 5 ng/ml IL-3. The changes of the proportion of GFP-positive cells with 5 ng/ml IL-3 (*A*) or 100 ng/ml SCF (*B*) for the indicated periods were assayed by flow cytometer. *C* and *D*, three independent Ba/F3-kit clones infected with wild-type or ΔC-Spred-1 cDNAs were plated in 96-well plates in RPMI 1640 medium containing various concentration of IL-3 (*C*) or SCF (*D*) and cultured for 72 h. Cell proliferation was measured by colorimetric assay using WST-1 and 1-methoxy phenazine methosulfate. The data shown are for three independent clones in each case, and *error bars* represent standard deviation for each experiment. For statistical analysis, the Student's *t* test was performed, and the *p* values are shown.

This may be due to large conformational changes by internal deletions. On the other hand, the N-terminal deletion mutant of Spred-1 (ΔN1), which lacks the EVH1 domain and internal region but contains the KBD and C-terminal SPR domain, strongly enhanced basal level of phosphorylation of ERK, although the precise mechanism of this phenomenon has not been clarified. However, overexpression of ΔC-Spred-1 did not much affect the basal level of ERK phosphorylation. Therefore, we further investigated the effect of ΔC-Spred-1 on ERK activation. As shown in Fig. 2, *C* and *D*, overexpression of wild-type Spred-1 strongly suppressed EPO- or G-CSF-induced ERK activation, whereas ΔC-Spred-1 augmented EPO- or G-CSF-induced ERK activation. These results suggested that ΔC-Spred-1 functions as a dominant negative form of Spred-1, which augments not only growth factor-induced but also cytokine-induced ERK activation.

Suppression of Proliferation of Hematopoietic Cells by Spred-1—We next examined the effect of wild-type Spred-1 and ΔC-Spred-1 on proliferation of Ba/F3-kit cells. Wild-type Spred-1 and ΔC-Spred-1 cDNAs were introduced into Ba/F3-kit cells with EGFP using a bicistronic retrovirus vector pMY-IRES-EGFP (21). Since the infected cells expressed both EGFP and FLAG-tagged Spred-1, the percentage of infected cells was determined as the EGFP-positive rate by flow cytometry. When Ba/F3-kit cells were cultured in a medium containing IL-3 for 3 weeks, the proportion of control IRES-EGFP-infected cells was not changed; however, the population of wild-type Spred-1-infected cells decreased, whereas that of ΔC-Spred-1-infected cells increased (Fig. 3*A*). Ba/F3-kit cells could survive 7 days at most in the presence of SCF without IL-3. As shown in Fig. 3*B*, SCF-dependent cell proliferation was also decreased by wild-type Spred-1 but increased to some extent by ΔC-Spred-1 infection.

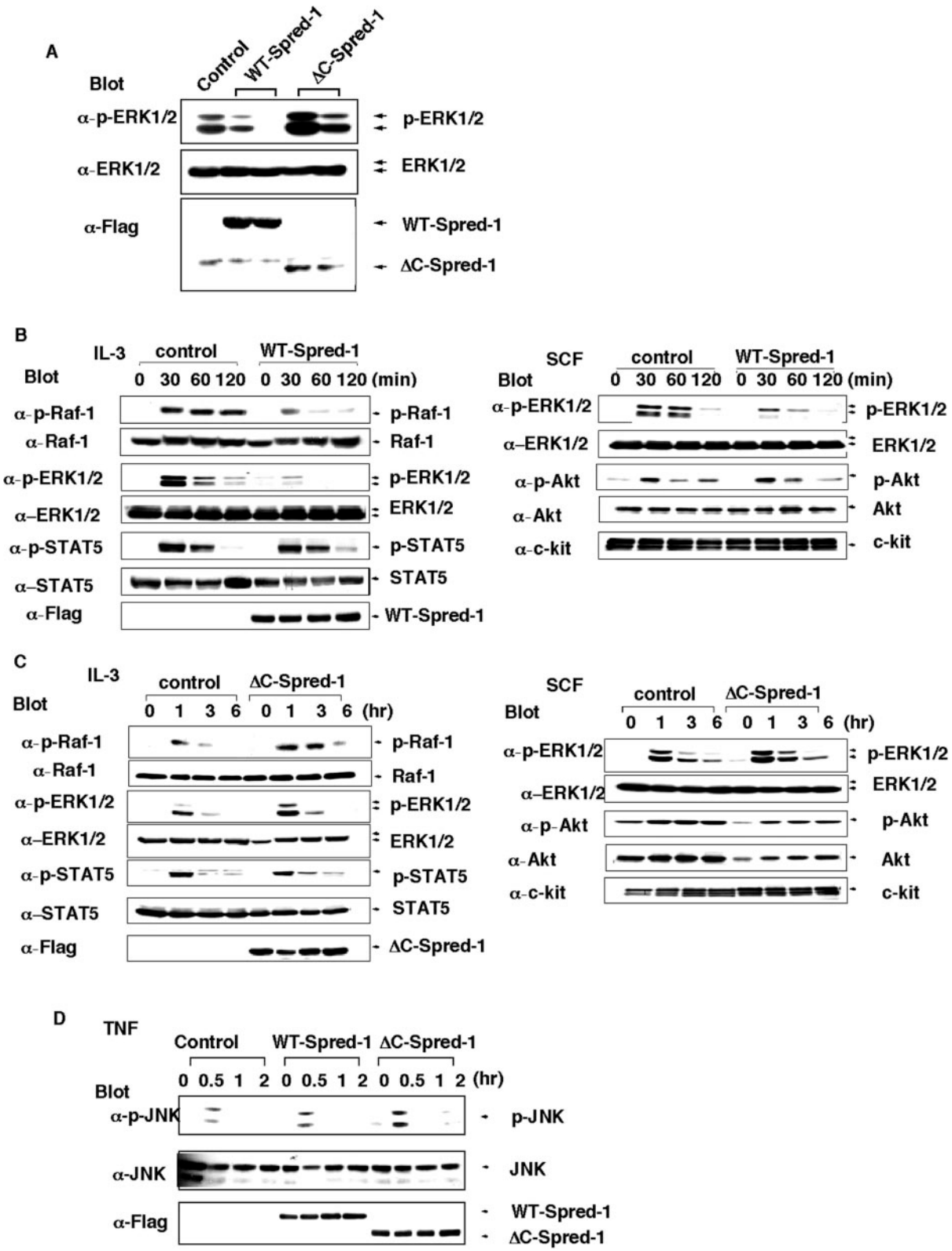

FIG. 4. **Effect of wild-type and ΔC-Spred-1 on activation of ERK in Ba/F3-kit cells.** *A*, control EGFP vector, wild-type Spred-1, or ΔC-Spred-1-infected Ba/F3-kit clones grown in normal condition in the presence of IL-3 were harvested, and cell extracts were immunoblotted with the indicated antibodies. *p-ERK1/2*, phosphorylated ERK1/2. *B–D*, control vector, wild-type Spred-1, or ΔC-Spred-1-infected Ba/F3-kit clones established by limiting dilution were stimulated with 50 ng/ml IL-3, 50 ng/ml SCF, or 20 ng/ml tumor necrosis factor for the indicated periods. Cell extracts were immunoblotted with the indicated antibodies. *p-Raf-1*, phosphorylated Raf-1; *p-Stat5*, phosphorylated Stat5; *p-Akt*, phosphorylated Akt; *p-Jnk*, phosphorylated JNK.

The suppressive and enhancing effects of wild-type Spred-1 and ΔC-Spred-1 on proliferation, respectively, were confirmed by infected Ba/F3-kit clones. Wild-type Spred-1- and ΔC-Spred-1-infected Ba/F3-kit cells were cloned by limiting dilution. The dose-response experiments to IL-3 and SCF using three independent clones revealed that WT-Spred-1 suppressed, whereas ΔC-Spred-1 significantly augmented cell growth (Fig. 3, *C* and *D*). These data indicate that Spred-1

inhibits not only growth factor-induced but also cytokine-induced proliferation.

Suppression of MAP Kinase Activation in Hematopoietic Cells by Spred-1—To explore the mechanism of the regulation of proliferation by WT- and ΔC-Spred-1 in Ba/F3-kit cells, we examined the level of ERK phosphorylation at steady state culture conditions with IL-3. As shown in Fig. 4*A*, ERK phosphorylation levels were lower in WT-Spred-1-infected clones, whereas they were higher in ΔC-Spred-1-infected clones than GFP-infected control clones. Thus, suppression and augmentation of proliferation by WT-Spred-1 and ΔC-Spred-1 shown in Fig. 3 could be due to the modulation of activation levels of ERK. Therefore, we examined the SCF- or IL-3-induced signaling pathways. In a wild-type Spred-1-infected clone, IL-3-induced ERK1/2 activation was suppressed as compared with the control, although STAT5 activation was not affected (Fig. 4*B*). SCF-induced ERK1/2 activation was also suppressed in wild-type Spred-1-infected cells, whereas Akt activation was not changed. On the other hand, ΔC-Spred-1 infection resulted in a slight but significant augmentation of IL-3- and SCF-induced ERK1/2 activation (Fig. 4*C*). We have shown that Spreds suppress ERK activation by inhibiting Raf, but not Ras, activation in response to growth factors (10). As shown in Fig. 4, *B* and *C*, WT-Spred-1 suppressed Raf-1 activation, whereas ΔC-Spred-1 enhanced it, as monitored by Ser-338 phosphorylation, further suggesting that Spred-1 inhibits ERK by suppressing Raf activation and ΔC-Spred-1 function as a dominant negative form of endogenous WT-Spreds.

To examine whether Spred-1-induced negative regulation is restricted to ERK, we investigated the effect of Spred-1 on the other MAP kinase, JNK. We stimulated Ba/F3 cells with tumor necrosis factor-α since JNK activation was marginal in response to SCF or IL-3 in Ba/F3 cells. As shown in Fig. 4*D*, Spred-1 did not affect tumor necrosis factor-α-induced JNK activation. Although we cannot completely rule out the possibility that Spred-1 affects other unknown pathways, these data suggest that wild-type Spred-1 suppresses the proliferation of Ba/F3-kit cells in the presence of IL-3 or SCF by suppressing ERK activation, whereas ΔC-Spred-1 augments proliferation through enhancing the ERK activity.

Effects of Spred-1 on Proliferation and Differentiation of Primary Bone Marrow Cells—To determine the function of Spred-1 in primary hematopoietic progenitor cells, we performed *in vitro* colony assays using mouse BM cells. After retrovirus carrying wild-type or ΔC-Spred-1 and EGFP was infected into mouse BM cells, GFP-positive cells were sorted by a fluorescence-activated cell sorter and plated in a methylcellulose culture medium containing various cytokines. More than 90% of the colonies expressed substantial levels of EGFP, as determined by fluorescence microscopy (data not shown). The numbers of CFU-GM and BFU-E from wild-type Spred-1-infected BM cells significantly decreased ($p < 0.01$) as compared with control EGFP-infected BM cells (Fig. 5*A*). Moreover, the sizes of the colonies derived from wild-type Spred-1-infected BM cells were generally less than one-fifth of those from EGFP-infected BM cells (Fig. 5*B*). On the other hand, the number of CFU-GM and BFU-E from ΔC-Spred-1-infected BM cells slightly increased ($p < 0.05$) as compared with EGFP-infected BM cells (Fig. 5*A*). These data suggest that Spred-1 negatively regulates hematopoiesis of bone marrow cells by suppressing ERK pathway.

Enhanced Proliferation and ERK Activation of Spred-1-deficient BMMCs—To further support our finding that Spred-1 regulates hematopoietic cytokine-induced ERK activation, we examined the effect of the loss of *Spred-1* using BMMCs from Spred-1 knockout mice. Spred-1 knockout mice were born normally.

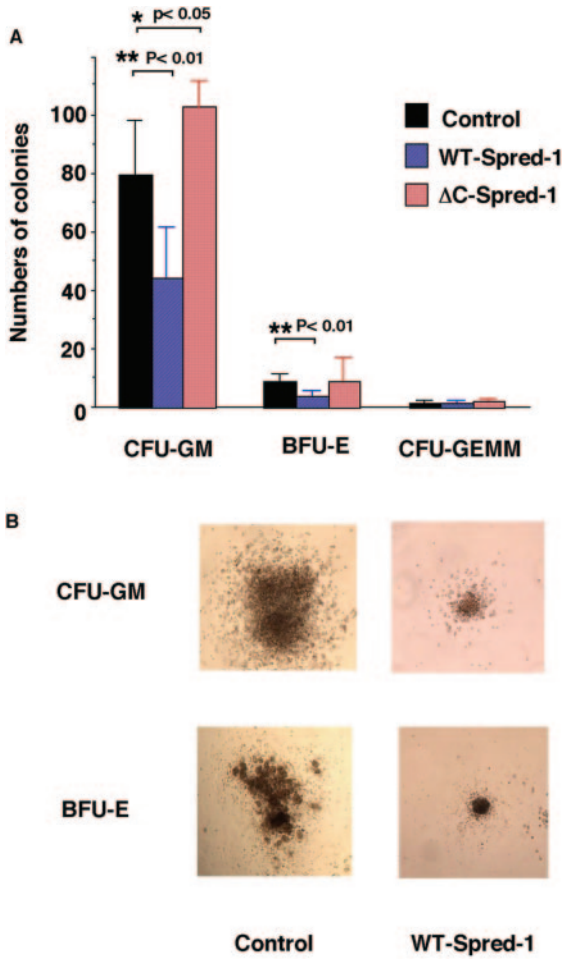

FIG. 5. **Effect of wild-type and ΔC-Spred-1 on colony formation of hematopoietic progenitors in bone marrow.** Retroviruses carrying wild-type and ΔC-Spred-1 cDNAs were infected into mouse bone marrow cells for 48 h. GFP-positive bone marrow cells were sorted, and 3×10^3 cells were plated in methylcellulose containing 5 ng/ml IL-3 or 50 ng/ml SCF and IL-6 and 3 units of EPO. *A*, on day 14, the number of colonies for CFU-GM, BFU-E, and CFU-GEMM were counted microscopically ($n = 4$; *error bars* represent standard deviation). For statistical analysis, the Student's *t* test was performed, and the *p* values are shown. *B*, typical appearance of CFU-GM (*top*) and BFU-E (*bottom*) of control-GFP (*left*) and wild-type Spred-1 (*right*)-infected cells are shown. Original magnification, ×100.

BMMCs were induced by culturing bone marrow cells with IL-3 for 4 weeks. As shown in Fig. 6*A*, high levels of Spred-1 protein expression were detected in Spred-$1^{+/+}$ BMMCs but not in Spred-$1^{-/-}$ BMMCs. SCF-induced ERK1/2 activation was sustained in Spred-$1^{-/-}$ BMMCs as compared with that of Spred-$1^{+/+}$ BMMCs. We then examined IL-3-induced signaling pathways. As shown Fig. 6*B*, IL-3-induced ERK1/2 activation as well as Raf-1 activation was augmented and prolonged in Spred-1-deficient BMMCs as compared with that of Spred-$1^{+/+}$ BMMCs, although JAK2 activation as well as STAT5 activation were not much affected. These data are consistent with our proposal that Spred-1 inhibits Ras/Raf/ERK pathway downstream of JAKs. IL-3-dependent growth of Spred-$1^{-/-}$ BMMCs was significantly faster than that of Spred-$1^{+/+}$ BMMCs (Fig. 6*C*). These data also suggest that Spred-1 negatively regulates the growth of BMMCs by regulating the activation induced by hemopoietic growth factors and cytokines.

DISCUSSION

ERKs are members of the MAP kinase family and have important roles in many facets of cellular regulation including cell proliferation, differentiation, and apoptosis. It has been

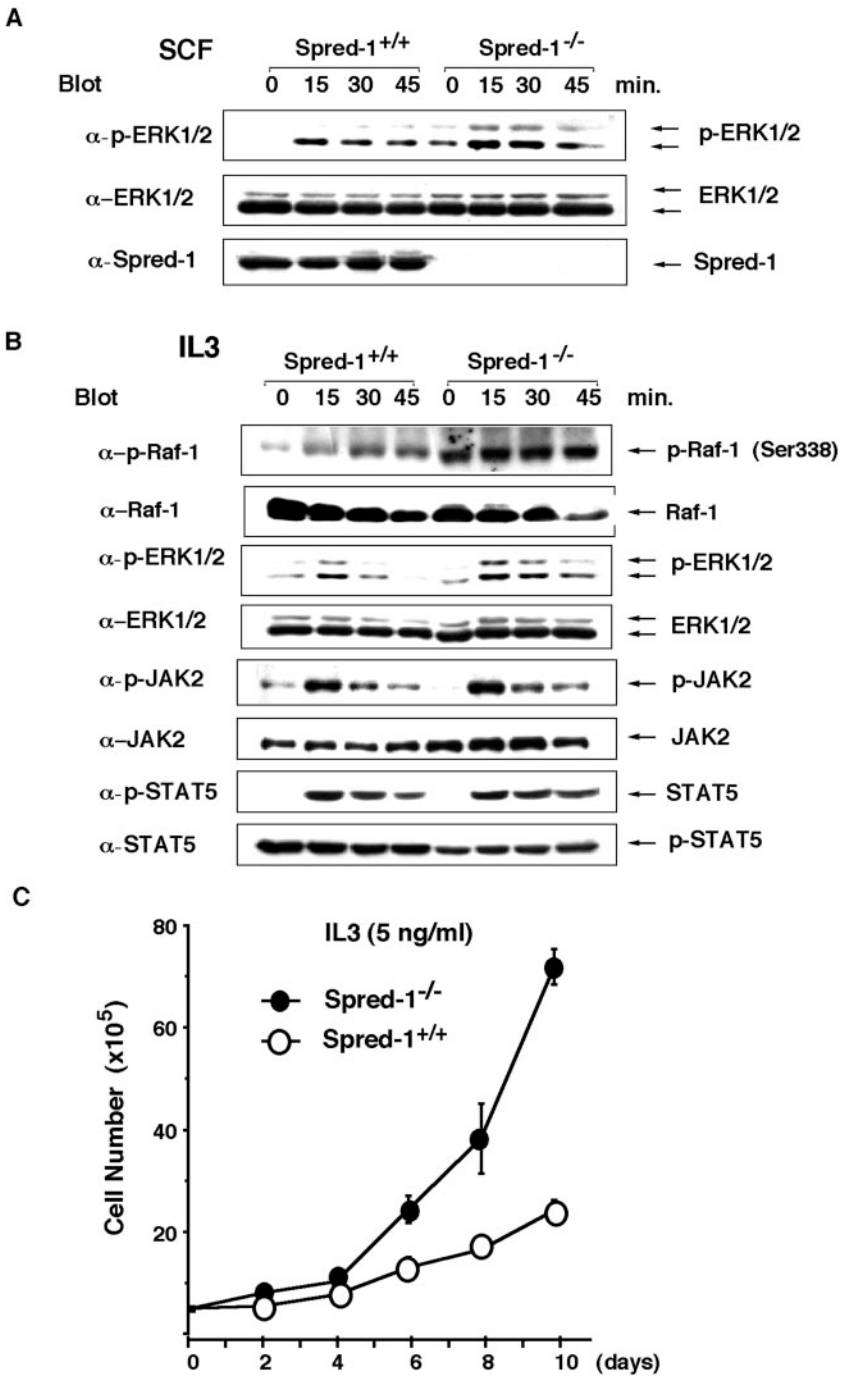

FIG. 6. **Effect of *Spred-1* gene disruption on cell proliferation and ERK activation in BMMCs.** *A* and *B*, BMMCs from Spred-$1^{+/+}$ and Spred-$1^{-/-}$ mice were stimulated with 50 ng/ml SCF (*A*) or 50 ng/ml IL-3 (*B*) for the indicated periods. Cell extracts were immunoblotted with the indicated antibodies. *p-ERK1/2*, phosphorylated ERK1/2; *p-Raf-1*, phosphorylated Raf-1; *p-JAK2*, phosphorylated JAK2; *p-Stat5*, phosphorylated Stat5. *C*, BMMCs (5×10^5 cells on day 0) from Spred-$1^{+/+}$ and Spred-$1^{-/-}$ mice were cultured in RPMI 1640 medium containing 5 ng/ml IL-3 for the indicated periods, and viable cell numbers were counted. *Error bars* represent standard deviation for three independent experiments.

well established that MAP kinase signaling pathways including ERK, p38, and JNK are activated by various cytokines and that growth factors are required in normal hematopoiesis (24). Among these MAP kinase groups, ERK is activated by hematopoietic cytokines such as EPO, IL-3, and SCF (25–28). Here we demonstrated that a novel ERK pathway inhibitor, Spred-1, can negatively suppress IL-3 as well as SCF-induced ERK activation without affecting STAT or Akt activation and can modulate cell proliferation. Since endogenous Spred-1 is expressed in hematopoietic cell lines and BMMCs, and ΔC-Spred-1 (a dominant negative form of Spreds) (Figs. 2–5) or deletion of the *Spred-1* gene (Fig. 6) can enhance the effect of IL-3 and SCF, Spred-1 is suggested to be a physiological negative regulator of hematopoietic growth factors and cytokines.

There are three members of the Spred family of proteins (17). Spred-2 is ubiquitously expressed, whereas Spred-1 expression is restricted in brain and heart, and Spred-3 is expressed only in the brain. In the present study, we showed that Spred-1 is also highly expressed in hematopoietic cells. However, Spred-1 is not expressed in all hematopoietic cells. Our reverse transcription-PCR analysis suggested that Spred-1 expression level was high in mast cells (BMMCs), erythroid cells, and B cells but low in megakaryocytes and macrophages.[2] Although we did not observe the rapid induction of Spred-1 protein expression in response to IL-3 and SCF in BMMCs, Spred-1 expression was strongly up-regulated during long term culture of BM cells in the presence of IL-3. This suggests that Spred-1 expression is restricted in a particular subset of hematopoietic cells or up-regulated during maturation of the cells. Therefore, unlike

[2] A. Nonami, unpublished data.

Sproutys, Spred-1 seems to be a developmentally regulated negative regulator rather than a negative feedback suppressor; it may be involved in regulation of the number and function of terminally differentiated cells. Interestingly, Spred-2 is shown to be expressed in AGM region, and overexpression of Spred-2 in the AGM culture significantly reduced the number of $CD45^+$ cells. In contrast, production of $CD45^+$ cells from the AGM region of Spred-2 null mice was up-regulated as compared with wild-type littermates (18). These data raise a possibility that Spred-2 functions as a negative regulator of embryonic hematopoiesis, whereas Spred-1 regulates the late phase of hematopoiesis by inhibiting hematopoietic cytokine signaling.

Recent studies have revealed many proteins that negatively modulate the Ras/MAPK pathway. These proteins can be roughly divided into two groups: phosphatases and scaffold proteins. The former includes MKP-1 (9), protein phosphatase 1 (PP1), and protein phosphatase 2A (PP2A) (30–32). The latter includes kinase suppressor of Ras (KSR), Sur8, connector enhancer of KSR (CNK), rat kinase inhibitor protein (RKIP), MP-1, RIN1, and the Sprouty/Spreds (33). Two other novel proteins that also suppress the MAP kinase pathway, Erbin (34) and Sef (35), have recently been reported. $P62^{dok}$ is an adaptor molecule that recruits Ras-GTPase activating protein (GAP), resulting in the suppression of the Ras/MAPK pathway, and hematopoietic cells from $P62^{dok}$ knockout mice proliferate more rapidly after growth factor stimulation with sustained MAPK activation than wild-type cells do (36). The knockout mice showed a shortened latency of the myeloproliferative disease induced by retroviral-mediated transduction of $p210^{bcr\text{-}abl}$ in bone marrow cells (36). Lnk is another adaptor protein mainly expressed in lymphocytes that negatively regulates MAP kinase activation (37, 38). The absence of Lnk resulted in an increase in the number and *in vitro* proliferative capacity of hematopoietic progenitor cells. Spred-1 may have a similar function to Dok and Lnk in hematopoietic cells. These studies also revealed the importance in hematopoiesis of the Ras/MAP kinase pathway and its regulatory functions.

The ERK pathway plays positive and negative roles in hematopoiesis. ERK1/2 activation has also been involved in mediating differentiation along the megakaryocytic and myeloid lineages. The ERK1/2 pathway antagonizes erythroid differentiation (39), whereas this pathway is necessary for GM-CSF-induced proliferation (40). Mutational analysis of G-CSF receptor indicates that ERK activation rather than STAT3 activation is required for proliferation (41). The ERK pathway has been also implicated directly and indirectly to play a critical role in megakaryopoiesis (42–44). For example, the MEK inhibitor PD98059 can increase megakaryocyte precursor number and delay megakaryocytic maturation of human umbilical cord $CD34^+$ cells cultured in thrombopoietin (42), and it can inhibit DNA polyploidization of murine bone marrow $CD41^+$ cells cultured in thrombopoietin (43). Furthermore, Raf-1 and B-Raf are shown to be required for thrombopoietin-induced sustained ERK1/2 activation, which itself is required for thrombopoietin-induced megakaryocytic maturation (44). From these observations, we speculate that Spred-1 as well as Spred-2 regulate proliferation and differentiation of hematopoietic lineages by modulating ERK activity. This is supported by the fact that a marked increase in the number of megakaryocytes was observed in the spleen of Spred-2-deficient mice (18) as well as Spred-1-deficient mice.[3] We also observed enhanced colony formation in Spred-1-deficient BM cells.[2] Rapid proliferation of Spred-1-deficient BMMCs and Ba/F3 cells expressing ΔC-Spred-1 in response to IL-3 also supports our hypothesis. Further investigation using knockout mice is under way to define which lineages of hematopoiesis and which cytokines Spreds regulate. If our hypothesis is also true in hematopoietic stem cells, inhibition of Spred function or suppression of expression of Spreds may be useful methods for the expansion of stem cells.

We have shown that Spred-1 interacts with Ras and Raf, thereby suppressing Raf-1 phosphorylation (10). We confirmed suppression of Raf-1 activation by Spred-1 using Ba/F3 cells (Fig. 4*C*) and Spred-$1^{-/-}$ BMMCs (Fig. 6*B*). Spred-1 seems to be a general inhibitor for the Ras/Raf/MAP kinase pathway, which is consistent with Spred-1 being able to suppress not only growth factor-induced but also cytokine-induced ERK activation. Although Sproutys are suggested to be selective inhibitors of ERK activation, we and others have shown that Sprouty-4 inhibits FGF and vascular endothelial growth factor (VEGF)-induced, but not EGF-induced, ERK activation (6, 20). The molecular basis of this selective suppression by Sproutys has not been clarified. Sproutys are also tyrosine-phosphorylated like Spred-1, and multiple mechanisms of suppression such as recruiting SHP-2 or Cbl are proposed (45). It is also not clear whether Sprouty and Spreds regulate signaling pathways other than ERK. In this context, we observed that overexpression of WT Spred-1 has a much more dramatic effect on IL-3- and SCF-induced ERK1/2 phosphorylation than does expression of ΔC-Spred-1 at short term stimulation (Fig. 4, *B* and *C*). This suggests a possibility of the effect of ΔC-Spred-1 on other signaling pathways. However, when we compared steady state phosphorylation levels of ERK (Fig. 4*A*), we found a more drastic effect of ΔC-Spred-1 on ERK activation. Therefore, a long term accumulation of enhanced ERK activation by ΔC-Spred-1 may contribute to the rapid proliferation of ΔC-Spred-1-infected cells.

However, *Xenopus* Sprouty2 is shown to be an intracellular antagonist of FGF-dependent calcium signaling (46). Recently, we and others showed that Spreds and Sproutys inhibit cell migration, probably by suppressing Rho activation (29, 47). Spred-1 interacted with Rho but not cdc42 and Rac and inhibits Rho-kinase activation (47). In addition, we found that Spred-1-deficient BMMCs can proliferate much faster than WT-BMMCs as ΔC-Spred-1 infected Ba/F3 cells. However, we noticed that such a rapid proliferation is dependent on culture periods. The difference in growth rate between WT and Spred-$1^{-/-}$ BMMC became small after more than 2 months of culture in IL-3, although Spred-1 was still expressed in WT-BMMCs and ERK activation was enhanced in Spred-$1^{-/-}$ BMMCs (data not shown). These cannot be explained by ERK alone, and we speculate that signaling pathways other than ERK also be affected by Spred-1 in hematopoietic cells. Further investigation is necessary to define the precise roles of Spred-1 on cytokine signal transduction.

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