Nf1 Regulates Hematopoietic Progenitor Cell Growth and Ras Signaling in Response to Multiple Cytokines

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Summary

Neurofibromin, the protein encoded by the *NF1* tumor-suppressor gene, negatively regulates the output of $p21^{ras}$ (Ras) proteins by accelerating the hydrolysis of active Ras-guanosine triphosphate to inactive Ras-guanosine diphosphate. Children with neurofibromatosis type 1 (NF1) are predisposed to juvenile chronic myelogenous leukemia (JCML) and other malignant myeloid disorders, and heterozygous *Nf1* knockout mice spontaneously develop a myeloid disorder that resembles JCML. Both human and murine leukemias show loss of the normal allele. JCML cells and $Nf1^{-/-}$ hematopoietic cells isolated from fetal livers selectively form abnormally high numbers of colonies derived from granulocyte-macrophage progenitors in cultures supplemented with low concentrations of granulocyte-macrophage colony stimulating factor (GM-CSF). Taken together, these data suggest that neurofibromin is required to downregulate Ras activation in myeloid cells exposed to GM-CSF. We have investigated the growth and proliferation of purified populations of hematopoietic progenitor cells isolated from *Nf1* knockout mice in response to the cytokines interleukin (IL)-3 and stem cell factor (SCF), as well as to GM-CSF. We found abnormal proliferation of both immature and lineage-restricted progenitor populations, and we observed increased synergy between SCF and either IL-3 or GM-CSF in $Nf1^{-/-}$ progenitors. $Nf1^{-/-}$ fetal livers also showed an absolute increase in the numbers of immature progenitors. We further demonstrate constitutive activation of the Ras-Raf-MAP (mitogen-activated protein) kinase signaling pathway in primary c-kit^{+} $Nf1^{-/-}$ progenitors and hyperactivation of MAP kinase after growth factor stimulation. The results of these experiments in primary hematopoietic cells implicate *Nf1* as playing a central role in regulating the proliferation and survival of primitive and lineage-restricted myeloid progenitors in response to multiple cytokines by modulating Ras output.

Key words: neurofibromin • hematopoietic progenitor • cytokines • Ras • granulocyte/macrophage colony–stimulating factor

Ras proteins regulate the growth and differentiation of many cell types by acting as molecular switches that transduce signals from the extracellular environment to the nucleus (1–9). The biochemical output of Ras proteins is tightly regulated by their ability to cycle between an active guanosine triphosphate (GTP)-bound state (Ras-GTP) and an inactive guanosine diphosphate (GDP)-bound state (Ras-GDP) (1–4). Ras activation is an essential component of proliferative responses induced after receptor binding by a variety of growth factors including IL-3, GM-CSF, CSF-1, and stem cell factor (SCF)[1] (5–9). Stimulation by each of these cytokines induces an increase in the percentage of Ras-GTP in the target cell (5, 6, 8, 9). Ras-GTP recruits Raf-1 to the plasma membrane and Raf-1, in turn, acti-

[1]*Abbreviations used in this paper:* CFC, colony-forming cells; GAP, GTPase-activating protein; HPP, high proliferating potential; JCML, juvenile chronic myelogenous leukemia; LPP, low proliferating potential; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase/extracellular signal-regulated kinase; NF1, neurofibromatosis type 1; SCF, stem cell factor.

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vates a series of downstream effectors such as mitogen-activated protein (MAP) kinase (7, 10, 11). GTPase-activating proteins (GAPs) regulate Ras output by stimulating the slow intrinsic Ras GTPase (4, 12, 13). Because it is Ras-GTP that transduces signals, GAPs act (at least in part) as negative regulators of Ras function (1–4, 12). Two GAPs, p120 GAP (also known as Ras-GAP) and neurofibromin (the protein encoded by the *NF1* gene) regulate Ras output in mammalian cells (4, 12–15) by promoting the conversion of Ras-GTP to Ras-GDP (4).

Mutations of *NF1* cause neurofibromatosis type 1 (NF1), an autosomal dominant disorder with an incidence of 1 in 4,000 (14–18). Affected individuals are predisposed to the development of benign and malignant neoplasms that arise primarily in cells derived from the embryonic neural crest (14–19). In addition, children (but not adults) with NF1 have a markedly increased risk of developing malignant myeloid disorders and comprise as many as 10% of de novo cases of preleukemia in the pediatric age group (19–24). Juvenile chronic myelogenous leukemia (JCML), a myeloproliferative syndrome characterized by leukocytosis, thrombocytopenia, and hepatosplenomegaly with infiltrates of myeloid cells, is the most common leukemia seen in children with NF1 (20–25). Genetic and biochemical data from studies of human leukemias have shown that *NF1* functions as a tumor suppressor gene in hematopoietic cells by negatively regulating Ras signaling (20, 22–25). Mice that are heterozygous for a targeted disruption of *Nf1* are predisposed to a number of cancers including myeloid leukemia (26). Leukemic cells from these animals show loss of the normal *Nf1* allele (26). Although mice homozygous for disruption of *Nf1* die during embryonic development, $Nf1^{-/-}$ fetal liver cells efficiently reconstitute hematopoiesis and consistently induce a JCML-like myeloproliferative syndrome in irradiated recipients (27).

A hallmark of low density blood and bone marrow cells from children with JCML is an abnormal pattern of in vitro progenitor growth that is characterized by the appearance of factor-independent myeloid colonies in methylcellulose cultures (28, 29). A second consistent finding is that JCML cells form higher numbers of colonies derived from lineage-restricted colony-forming unit granulocyte-macrophage progenitors (CFU-GM) in the presence of low concentrations of GM-CSF (21, 28, 29). Hypersensitive growth has not been observed in cultures in which IL-3 or GM-CSF was added, and these results have led to the hypothesis that a specific defect within the GM-CSF signal transduction pathway plays a central role in the pathogenesis of JCML (28, 29). $Nf1^{-/-}$ fetal liver cells also demonstrate a similar pattern of selective GM-CSF hypersensitivity in myeloid progenitor assays (27, 30) and provide a model system to investigate both the role of neurofibromin in regulating Ras signaling in hematopoietic cells and also the pathogenesis of JCML.

We have used *Nf1* mice to address two questions related to the role of neurofibromin in regulating hematopoietic cell growth through the Ras-MAP kinase pathway. First, since the hematopoietic system is hierarchial and has multiple compartments, it is possible that neurofibromin is only required to regulate growth of specific subpopulations of cells in response to a restricted number of hematopoietic growth factors. Previous studies of human and murine cells examined only the responsiveness of relatively differentiated, lineage-restricted progenitors from mixed populations of hematopoietic cells, and in most instances cultures were established using single growth factors (21, 27, 29–33). We isolated highly pure hematopoietic progenitors and examined their growth in response to GM-CSF, IL-3, and stem cell factor (SCF) singly and in combination with other factors. SCF promotes the survival and proliferation of immature, multilineage hematopoietic progenitors (34–37) and acts synergistically with growth factors, such as GM-CSF, that primarily affect differentiated, lineage-restricted cells (38–41). Second, the selective hypersensitivity of $Nf1^{-/-}$ and JCML cells to GM-CSF in colony-forming assays is perplexing because GM-CSF and IL-3 both increase Ras-GTP levels in cell lines (8, 9) and the cell surface receptors for these ligands share a common β chain that transduces signals (for review see references 42–44). To address these questions, we measured constitutive and cytokine-stimulated MAP kinase activation in well-defined populations of primary $Nf1^{-/-}$ and $Nf1^{+/+}$ hematopoietic cells and correlated these data with in vitro colony-forming assays that assessed the size and growth factor responsiveness of these populations. Our results implicate neurofibromin as regulating the size and growth factor responsiveness of both immature and lineage-restricted hematopoietic progenitor populations by modulating Ras output in response to multiple cytokines.

Materials and Methods

Isolation of Fetal Hematopoietic Cells. Heterozygous $Nf1^{+/-}$ mice were mated to produce $Nf1^{-/-}$ embryos. Pregnant $Nf1^{+/-}$ females were killed by cervical dislocation on day 13.5 of gestation and the embryos were removed through an anterior abdominal incision. After individual embryos were isolated, each fetal liver was removed and transferred to a 10 × 35 mm dish in IMDM supplemented with 20% FCS. A single cell suspension was prepared by pushing the hepatic tissues through progressively smaller needles (16–27 gauge). This procedure yielded 1–1.5 × 10^7 mononuclear cells.

Genotyping Fetal Tissues. Once the liver was removed, the remainder of the embryonic tissues were used for genotype analysis. Genomic DNA from fetal tissues were isolated as previously described (30). An assay based on the PCR was used to perform genotype analysis. The targeting vector used to disrupt the murine *Nf1* gene truncates exon 31 and inserts a neomycin resistance gene (*neo*). The 3′ (reverse) primer is complimentary to a sequence in the 3′ region of exon 31 that is present in both wild-type and targeted *Nf1* genes. Two 5′ primers were designed to distinguish disrupted and wild-type genes. The first is complimentary to *neo* and the second is complimentary to a wild-type exon 31 sequence. Amplification with the *neo* and 3′ *Nf1* primer pair yields a 350-bp product, whereas amplification with the 5′ *Nf1* and 3′ *Nf1* pair gives a 230-bp fragment. The sequences for

the PCR primers used in these studies are: 5′ *Nf1* exon 31: CACCTTTGTTTGGAATAT, 3′ *Nf1* exon 31: TTCAATACCTGCCCAAGG, and *neo*: ATTCGCCAATGACAAGAC.

Transplantation into Irradiation-conditioned Congenic Mice. Unfractionated fetal liver cells (1–2 × 10^6) from $Nf1^{-/-}$ or $Nf1^{+/+}$ littermates were transplanted into C57Bl/6J recipient mice conditioned with 1,100 rads of gamma irradiation from a Cs source (Gamma cell 40 Extractor; MDS Nordion Kanata, Ontario, Canada) with 700 rads given in the first dose and 400 rads given 4 h later. Recipients of $Nf1^{-/-}$ cells consistently develop myeloproliferative hematopoiesis with large numbers of mature neutrophils and monocytes in the peripheral blood and prominent splenomegaly (27). Bone marrow and splenic cells were obtained from recipients 18–30 wk after transplantation. Hematopoietic cells were evaluated at death by PCR to confirm the detection of only donor-derived cells.

Purification of Hematopoietic Progenitors. Low density cells were incubated with a PE-conjugated c-kit monoclonal antibody or a PE-conjugated $Sca1^+$ monoclonal antibody whose corresponding Ly6A/E antigen is highly expressed on all primitive hematopoietic progenitors and cells in the stem cell compartment in C57Bl6 strain (45). Cells were incubated with 1 μg of c-kit or Sca1 antibody/10^6 cells, (PharMingen, San Diego, CA), and the cells were then incubated on ice for 20 min, pelleted, washed, repelleted, and then suspended in FACS buffer. Cells were also simultaneously stained with antibodies to the following lineage markers including: anti-B220, anti-CD3ϵ, anti–Gr-1, and anti–Mac 1 all conjugated to FITC (PharMingen). They were then separated by FACS® using a dual laser FACStar PLUS® equipped with an argon and a krypton laser (Becton Dickinson, Mountain View, CA).

Hematopoietic Progenitor Colony Growth. Recombinant GM-CSF, IL-3, and SCF were obtained from Peprotech (Rocky Hill, NJ); recombinant mIL-1α and M-CSF were purchased from R&D Research Laboratories (Minneapolis, MN). Purified hematopoietic cells were placed into culture in triplicate 35-mm plates at a final concentration of 5 × 10^2 cells per ml. Cells and recombinant growth factors were added to the methylcellulose medium, (for growth of CFU-GM) or agar (for growth of low proliferating potential [LPP] colony-forming cells [CFC] and high proliferating potential [HPP]-CFC) and the solution was thoroughly admixed before plating as previously described (30, 46, 47). Cells cultured for growth of CFU-GM were maintained at 37°C in a humidified 5% CO_2 incubator 95% O_2 and scored on day 10 of culture. Growth factors used for culture of HPP-CFC and LPP-CFC included SCF, IL-1α, CSF-1, and GM-CSF or IL-3. Cultures for growth of LPP-CFC were cultured in 8% CO_2, 5% O_2, and were scored on day 7 of culture, whereas those for HPP-CFC were scored on day 14 of culture. Colonies were scored by indirect microscopy.

Kinase Assays. The incorporation of phosphate into myelin basic protein (MBP) (Sigma Chemical Co., St. Louis MO) was used as a measure of MAP kinase activity as previously described (48–51). Cell pellets were lysed in lysis buffer (10 mM K_2HPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3 $V0_4$, 50 mM β-glycerophosphate, 1% NP-40, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μg/ml PMSF) at 4°C, disrupted in 1 ml of Dounce (Kontes, Vineland, NJ), iced for 30 min, and clarified by ultracentrifugation at 100,000 *g* for 30 min at 4°C. Total protein was determined using a BCA protein assay kit (Pierce, Rockford, IL). The MBP reaction conditions consisted of 10 μl of cell lysate with 5 μg of MBP substrate and was incubated with 20 mM Tris-Hcl pH 7.25, 10 mM $MgCl_2$, 500 μM unlabeled ATP, 20 μg BSA, and 5 μCi γ-[^{32}P]ATP in a total reaction volume of 30 μl, at 30°C × 15′. Three replicates of each reaction were evaluated in each experiment. The reactions were stopped by the addition of 7.5 ml of 5× sample buffer and were then boiled for 2 min. The kinase reaction mixtures were resolved on 12.5% SDS-PAGE gels. Gels were then stained in a 0.2% Coomassie brilliant blue (Boehringer Mannheim, Indianapolis, IN) solution, dried on Whatman paper, and exposed to Kodak x-OMAT AR film (Eastman-Kodak Co., Rochester, NY) at −20°C. In addition to autoradiography, radiolabeled phosphate in MBP gel bands was also determined by liquid scintillation counting of the MBP bands. The cpm value was then used to calculate MAP kinase activity in pmol phosphate incorporated into MBP/min/μg protein in the cell lysate.

Liquid Cultures. Purified populations of c-kit^+ cells isolated from the bone marrows of mice transplanted with $Nf1^{-/-}$ or $Nf1^{+/+}$ cells were treated with the MAP kinase/ERK (extracellular signal–regulated) kinase (MEK) inhibitor PD 98059 (New England Biolabs, Beverly, MA) to a final concentration of 50 μM as previously described (52). All cultures were maintained at a density of 2 × 10^5 cells/ml in IMDM supplemented with 20% FCS. Cells were cultured in 100 ng/ml rmSCF and 50 U/ml rmIL-3, which is the maximum stimulating activity of each respective cytokine.

Results

Previous studies in JCML bone marrow samples have implicated cytokine production by accessory cells as contributing to the hypersensitive pattern of myeloid progenitor growth (31, 32). We therefore isolated highly pure (95–99%) $Sca1^+lin^{-/dim}$ cells derived from day 13.5 fetal livers by flow cytometric cell sorting and assayed the growth of CFU-GM in methylcellulose medium supplemented with varying concentrations of either GM-CSF or IL-3. A representative FACS® analysis of cells before and after cell sorting is shown in Fig. 1. The results of four independent experiments indicate that CFU-GM derived from $Nf1^{-/-}$ fetal liver $Sca1^+lin^{-/dim}$ cells retain a hypersensitive pattern of colony formation in response to GM-CSF but not IL-3

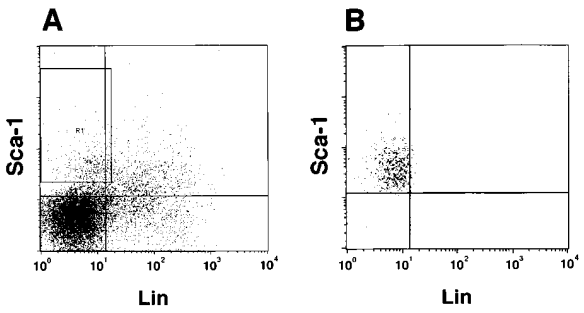

Figure 1. Isolation of $Sca1^+lin^{-/dim}$ hematopoietic progenitors from fetal liver cells. Pooled day 13.5 gestation fetal liver cells from each respective genotype were prepared for culture by isolation of low density cells using a Ficoll-Hypaque gradient. Low density cells were simultaneously stained with isotype controls or antibodies to Sca1 (PE) and lineage markers including anti-B220 (pan–B lymphocytes), anti-CD3ϵ (pan–T cells), anti–Gr-1 (granulocytes), and anti–Mac 1 (monocyte-macrophages) all conjugated to FITC. $Sca1^+lin^{-/dim}$ cells were identified relative to isotype control antibodies in the upper left corner of the dual parameter dot plot in *A*. These cells were isolated by fluorescence-activated cell sorting and a sample of sorted cells was reanalyzed to confirm the purity of isolated cells (*B*).

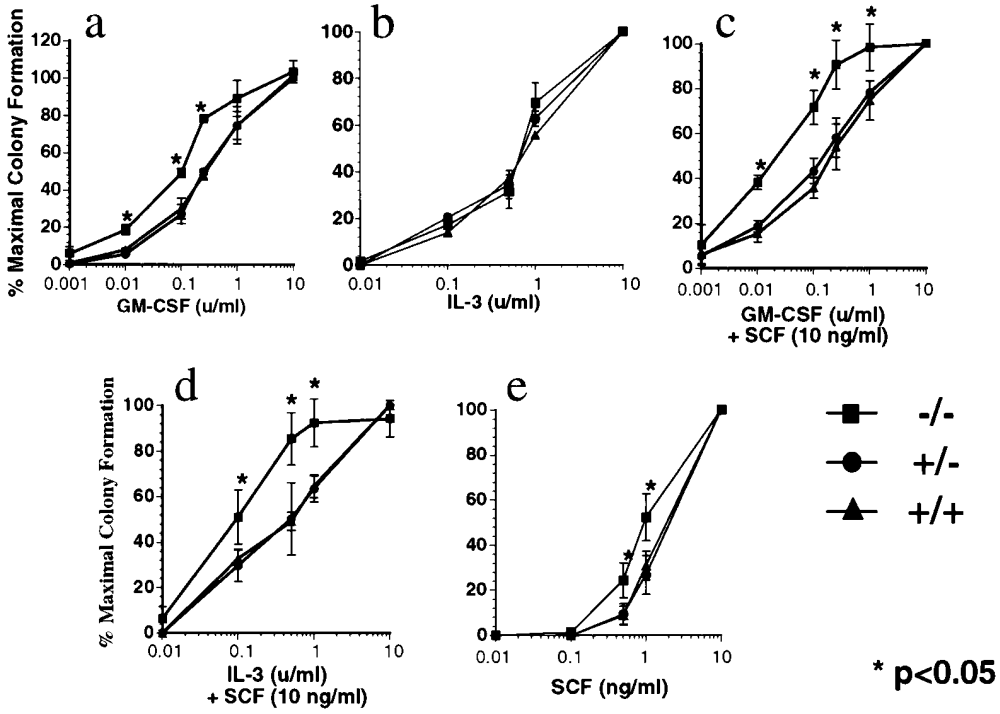

Figure 2. Dose–response curves of progenitor colony growth in response to cytokines. Growth of $Sca1^{+}lin^{-/dim}$ cells from wild-type ($Nf1^{+/+}$), heterozygous ($Nf1^{+/-}$), and homozygous ($Nf1^{-/-}$) mice is shown at the indicated concentrations of mGM-CSF (*a*), mIL-3 (*b*), mGM-CSF and 10 ng/ml SCF (*c*), mIL-3 and 10 ng/ml mSCF (*d*), or mSCF (*e*). The data are expressed as percentage of maximal numbers of CFU-GM colonies.

(Fig. 2, *a* and *b*). These data suggest that the alteration of growth responsiveness to GM-CSF seen in $Nf1^{-/-}$ cells is intrinsic to the progenitor compartment.

We next examined the effects of adding a low concentration of murine SCF (10 ng/ml) in combination with either GM-CSF or IL-3 on $Sca1^{+}lin^{-/dim}$ cells on colony formation. CFU-GM colony growth under these conditions is shown in Fig. 2, *c* and *d*, and the absolute numbers of progenitor colonies enumerated at maximal concentrations of GM-CSF or IL-3 singly and in combination with SCF are shown in Table 1. As with GM-CSF alone, the dose–response curve for CFU-GM colony formation from $Nf1^{-/-}$ cells was significantly left-shifted relative to $Nf1^{+/+}$ cells in cultures stimulated with GM-CSF + SCF (Fig. 2 *c*). The hypersensitive pattern of growth seen in $Nf1^{-/-}$ cells was more pronounced in parallel cultures stimulated with GM-CSF and SCF than in plates containing GM-CSF alone, and there was an increase in the absolute number of progenitors enumerated (Table 1). Surprisingly, the addition of SCF to cultures stimulated with IL-3 induced a hypersensitive pattern of growth in $Nf1^{-/-}$ cells (Fig. 2 *d*) that was not seen with IL-3 alone (Fig. 2 *b*). Although SCF is relatively inefficient at inducing colony formation in the absence of other growth factors (Table 1), we also detected a significant left shift in the dose–response curve of $Nf1^{-/-}$ versus $Nf1^{+/+}$ cells cultured in the presence of SCF alone (Fig. 2 *e*). Taken together, these data suggest that loss of *Nf1* deregulates the growth of hematopoietic progenitors in response to SCF, which acts on more immature populations of progenitors than do IL-3 or GM-CSF, and demonstrate that SCF can synergize with either factor to induce a hypersensitive pattern of in vitro growth in $Nf1^{-/-}$ progenitors.

One potential explanation for the hypersensitive growth seen in response to SCF alone or in combination with IL-3 is that SCF stimulation induces autocrine production of GM-CSF by $Nf1^{-/-}$ progenitor cells. To test this hypothesis, an anti–GM-CSF neutralizing antibody was added to the methylcellulose cultures stimulated with a combination of IL-3 and SCF. In pilot experiments, a concentration of anti–GM-CSF antibody that completely blocked CFU-GM colony formation in response to 10 U/ml of GM-CSF (maximum stimulating activity) was established and used

Table 1. *Maximal CFU-GM/5 × 10^2 $Sca1^{+}/lin^{-/dim}$ in Response to Cytokine Stimulation*

	Genotype		
Cytokine	−/−	+/−	+/+
GM-CSF (10 ng/ml)	71 ± 8.5	70 ± 11.2	68 ± 7.5
IL-3 (50 U/ml)	73 ± 15.6	68 ± 14.4	80 ± 14.3
GM-CSF (10 ng/ml) + SCF (10 ng/ml)	119 ± 23.0	89 ± 15.6	96 ± 12.3
IL-3 (50 U/ml) + SCF (10 ng/ml)	117 ± 20.8	110 ± 12.7	105 ± 15.6
SCF (100 ng/ml)	57 ± 16.1	47 ± 6.5	46 ± 8.3

The colonies enumerated from each respective genotype after stimulation with cytokines that yielded maximum colony activity. (See Fig. 2). Results are expressed as mean ± SE. All data represent analysis from four independent experiments.

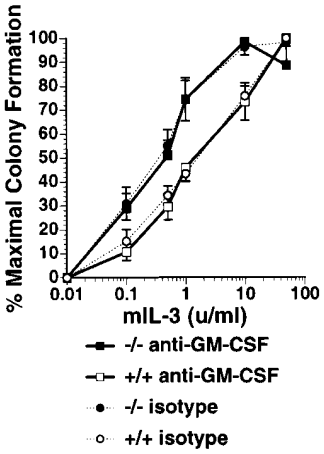

Figure 3. Progenitor colony growth in response to varying concentrations of IL-3 and SCF in the presence of neutralizing anti–GM-CSF antibodies. Growth of Sca1$^+$lin$^{-/dim}$ from wild-type (*Nf1*$^{+/+}$) and homozygous (*Nf1*$^{-/-}$) mice is shown at the indicated concentrations of mIL-3 and 10 ng SCF in the presence of anti–GM-CSF neutralizing antibodies or isotype control antibodies.

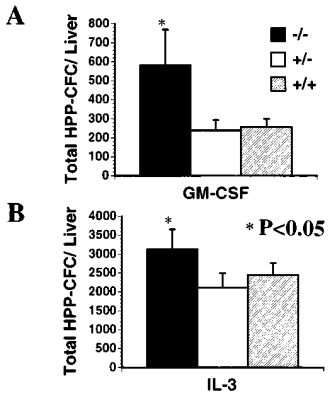

Figure 4. Growth of colonies derived from primitive progenitors in fetal livers. Total numbers of HPP-CFC from wild-type (*Nf1*$^{+/+}$), heterozygous (*Nf1*$^{+/-}$), and homozygous (*Nf1*$^{-/-}$) fetal livers are shown after culture in saturating concentrations of SCF, M-CSF, IL-1α, and either GM-CSF (*A*) or IL-3 (*B*).

in subsequent experiments (data not shown). Fig. 3 summarizes the results of three independent experiments examining the number of CFU-GM colonies seen when either anti–GM-CSF or an isotype control antibody was added to cultures containing IL-3 + SCF. These data indicate that the abnormal pattern of CFU-GM colony formation observed in *Nf1*$^{-/-}$ Sca1$^+$lin$^{-/dim}$ cells is maintained in the presence of anti–GM-CSF antibody, and therefore we conclude that hypersensitive growth in the presence of IL-3 + SCF is not due to autocrine GM-CSF production by *Nf1*$^{-/-}$ cells.

The experiments described above assayed the growth of colonies derived from CFU-GM, a relatively differentiated, lineage-restricted population of myeloid progenitors. However, data from cultures stimulated with SCF suggested that neurofibromin might also regulate the growth of more primitive hematopoietic compartments. To investigate this possibility, two-layer agar cultures were established to assay the growth of primitive progenitors with significant replating potential indicative of a primitive stem/progenitor cell (46) and referred to as HPP-CFC. Fetal liver cells from *Nf1*$^{-/-}$, *Nf1*$^{+/-}$, and *Nf1*$^{+/+}$ embryos were cultured in two different combinations of four growth factors at saturating activities to enumerate HPP-CFC. The frequency of HPP-CFC observed in cultures were then multiplied by the number of low density cells per organ to calculate the absolute number of HPP-CFC per fetal liver. The results shown in Fig. 4 demonstrate that significantly higher numbers of HPP-CFC are present from the *Nf1*$^{-/-}$ fetal liver cells. The data suggest that the loss of *Nf1* results in an expansion of HPP-CFC in vivo in the fetal liver and that neurofibromin is important in modulating the growth of primitive myeloid progenitor compartments as well as CFU-GM progenitors.

Stimulation with a variety of growth factors including SCF, GM-CSF, or IL-3 activates the Ras-Raf-MAP kinase cascade in cultured cell lines (5–9). We hypothesized that if neurofibromin is critical to regulate Ras signaling in response to these cytokines in primary hematopoietic progenitors, then loss of *Nf1* function might result in an augmented MAP kinase activity after stimulation. To test this possibility, c-kit$^+$ cells were isolated from the bone marrows of mice previously irradiated and then reconstituted with fetal liver cells from day 13.5 gestation *Nf1*$^{-/-}$ and *Nf1*$^{+/+}$ littermates. In pilot experiments, we determined that the c-kit$^+$ cells isolated from transplant recipients had a similar hyperresponsiveness to GM-CSF in clonogenic assays as did *Nf1*$^{-/-}$ fetal liver cells (data not shown). After isolation using fluorescence-activated cell sorting, c-kit$^+$ cells were maintained in liquid cultures overnight without exogenous growth factors. The cells were then stimulated with either SCF, IL-3, or GM-CSF alone or with a combination of SCF + IL-3. MAP kinase activity was measured in unstimulated cells as well as at 5 and 60 min after the addition of growth factor(s). Three independent experiments yielded similar results; data from one of these is shown in Fig. 5. A threefold elevation in MAP kinase activity was

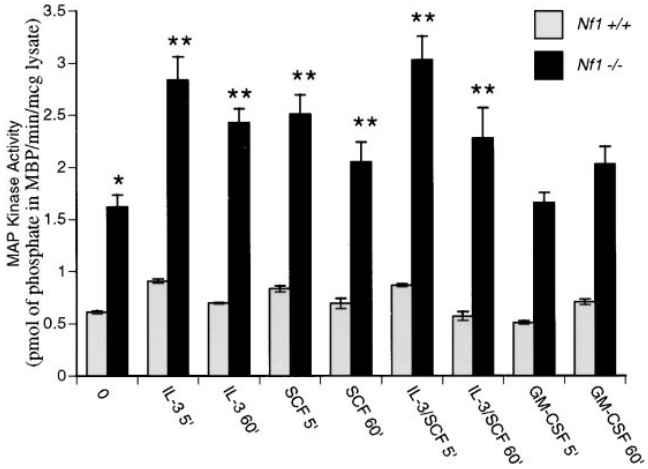

Figure 5. MAP kinase activity in c-kit$^+$ hematopoietic cells. c-kit$^+$ bone marrow cells from mice previously reconstituted with *Nf1*$^{-/-}$ or *Nf1*$^{+/+}$ fetal liver cells were isolated, stimulated with cytokines for specified times, and harvested, and cell lysates were prepared. MAP kinase activity was measured in whole cell extracts. Activity was calculated by determining the rate of incorporation of phosphate in MBP per min/μg of lysate and is indicated in the graph for each genotype, cytokine, and length of stimulation. The genotype and length of stimulation are indicated.

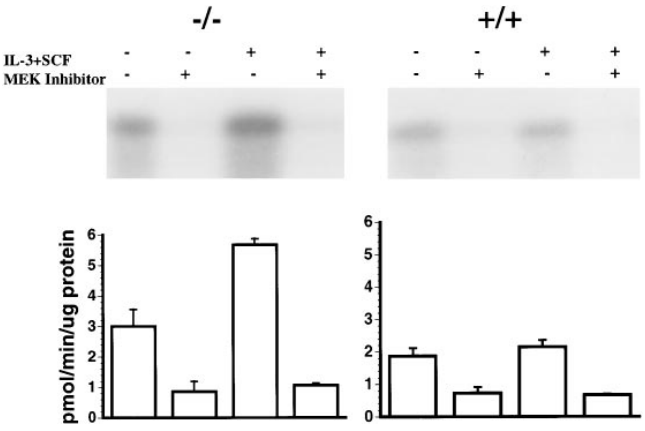

Figure 6. Inhibition of MAP kinase activity in $Nf1^{-/-}$ c-kit$^+$ cells exposed to MEK inhibitor before stimulation with cytokines. c-kit$^+$ bone marrow cells from mice reconstituted with $Nf1^{-/-}$ fetal liver cells (*left*) or $Nf1^{+/+}$ (*right*) were exposed to a MEK inhibitor or vehicle control before stimulation with a maximum stimulating capacity of IL-3 and SCF. Cells were lysed 5 min after stimulation with cytokines. The autoradiographs and activities of one of three independent experiments is shown. The cytokine and MEK inhibitor treatment of cells is indicated by + or −.

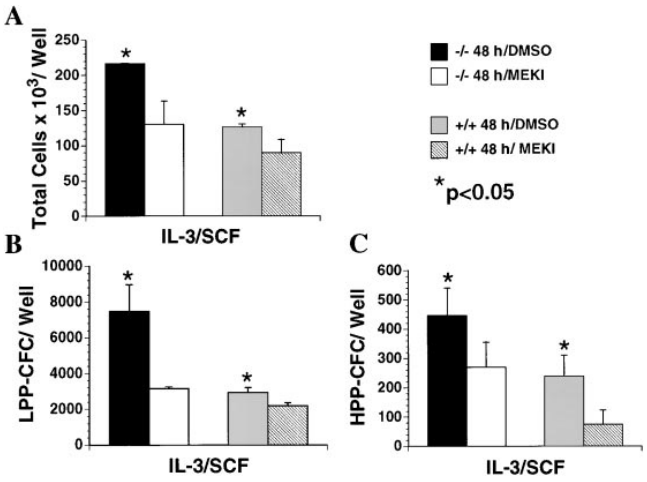

Figure 7. Effect of preincubation with an MEK inhibitor on progenitor colony formation from $Nf1^{-/-}$ cells. c-kit$^+$ bone marrow cells (4×10^4) were cultured with SCF and IL-3 for 48 h in the presence of the MEK inhibitor (*MEKI*) or the vehicle control (*DMSO*). After liquid culture, progenitor assays for growth of HPP-CFC and LPP-CFC were established. The total number of nucleated cells (*A*), LPP-CFC (*B*), and HPP-CFC (*C*) after culture for each genotype and condition are indicated. Based on cultures established in agar before treatment with the MEK inhibitor, 1,850 LPP-CFCs ± 10 SEM and 260 HPP-CFC ± 40 SEM per well were present in the input $Nf1^{-/-}$ cells, and 825 LPP-CFC ± 205 SEM and 100 HPP-CFC ± 40 SEM were present in input $Nf1^{+/+}$ cells.

observed in unstimulated c-kit$^+$ cells isolated from the $Nf1^{-/-}$ recipients. Kinase activity increased significantly in $Nf1^{-/-}$ cells after exposure to IL-3, SCF, or SCF + IL-3, and persisted above baseline at 60 min. There was also an increase in MAP kinase activity at 60 min after stimulation with GM-CSF that approached statistical significance. In contrast, c-kit$^+$ cells from the $Nf1^{+/+}$ recipients had a much less pronounced increase in MAP kinase activity at 5 min after stimulation, and the kinase returned to baseline by 60 min. We conclude that absence of neurofibromin in c-kit$^+$ hematopoietic progenitors results in a constitutive activation of Ras-MAP kinase signaling that can be further stimulated in response to SCF, IL-3, and GM-CSF.

To confirm an association between the clonogenic and biochemical data presented above, c-kit$^+$ cells were again isolated from transplant recipients. MAP kinase activity was determined on aliquots of $Nf1^{+/+}$ and $Nf1^{-/-}$ cells after overnight incubation in the presence or absence of an inhibitor of MEK. The remaining cells were either immediately cultured in methylcellulose to score the growth of myeloid progenitor colonies or were placed into liquid culture containing SCF and IL-3 together with MEK or the vehicle control for 48 h before plating. Biochemical data from one of three representative experiments are shown in Fig. 6 and the colony numbers are presented in Fig. 7. Addition of the MEK inhibitor reduced the MAP kinase activity of unstimulated $Nf1^{-/-}$ cells to wild-type levels and blocked kinase activation in response to SCF and IL-3. Similarly, addition of the MEK inhibitor to liquid cultures of c-kit$^+$ cells for 48 h before plating in methylcellulose markedly decreased the growth of differentiated cells, differentiated progenitor cells (LPP-CFC), and primitive hematopoietic progenitors (HPP-CFC) as compared with cultures containing the vehicle only. A reduced biochemical and cellular response to the MEK inhibitor was noted in the $Nf1^{+/+}$ hematopoietic cells. Taken together, these data implicate deregulated signaling through the Ras-MEK-MAP kinase in the aberrant pattern of cytokine-induced hematopoietic progenitor colony growth seen in $Nf1^{-/-}$ cells.

Discussion

Ras signaling plays a central role in normal myelopoiesis and is deregulated by at least three distinct genetic mechanisms in myeloid leukemia (for review see references 20, 53, 54). Ligand-induced activation of many hematopoietic growth factor receptors transiently increases the Ras-GTP levels, which in turn influences the survival, proliferation, and differentiation of myeloid-lineage cells. In leukemic cells, acquired mutations of *NRAS* or *KRAS*, formation of a chimeric protein as a result of *BCR-ABL* translocations, and inactivation of *NF1* all deregulate Ras signaling. Lines of knockout mice provide novel model systems for ascertaining how loss of function of specific genes influences complex biologic processes such as hematopoiesis and leukemogenesis. Although inactivation of the murine homologues of many human tumor suppressor genes results in a spectrum of neoplasms that differs significantly from the respective human cancers, *Nf1* mice spontaneously develop a type of leukemia that closely resembles JCML and $Nf1^{-/-}$ cells display a similar pattern of GM-CSF hypersensitivity in myeloid colony-forming assays.

A distinct advantage of this murine model is the ability

to reconstitute irradiated mice only lacking *Nf1* and isolate highly purified populations of phenotypically defined cells to characterize their in vitro colony growth and intracellular signaling.

This study addressed two questions: (*a*) what is the role of neurofibromin in modulating the cytokine responsiveness and growth of both primitive myeloid progenitors with multilineage and replating potential, and of more differentiated lineage-restricted myeloid progenitors?, and (*b*) how does inactivation of *Nf1* affect constitutive and cytokine-stimulated intracellular signaling through the Ras-Raf-MAP kinase pathway in these primary hematopoietic cells? We used antibodies to purify immature hematopoietic cells and focused on the SCF/c-kit signaling pathway because the binding of SCF to c-kit increases Ras-GTP levels and activates Raf-1 and MAP kinase activity in myeloid cell lines (7). In addition, the presence of multiple hematopoietic defects in lines of mice with mutations affecting either c-kit (dominant white spotting, *W*) or SCF (*Steel*) (34–37, 39, 55, 56), as well as numerous in vitro studies showing a synergistic effect between SCF and other cytokines in promoting the survival and proliferation of primitive hematopoietic progenitor cells, indicate that the SCF/c-kit signaling plays an important role in early hematopoiesis (38–41).

An intriguing finding demonstrated here was that SCF alone or in combination with IL-3 induced a hypersensitive pattern of CFU-GM colony growth in $Nf1^{-/-}$ cells. Though some reports using JCML cells have observed hypersensitive colony growth in response to only GM-CSF, others have inferred the involvement of other cytokines in the pathogenesis of the myeloproliferative disease (31–34, 57). Our results address an interesting paradox: since the GM-CSF and IL-3 receptors share a common β signaling chain that has been implicated in Ras activation, it has been perplexing why cultured $Nf1^{-/-}$ cells are hypersensitive to GM-CSF but not IL-3 in progenitor colony-forming assays. One explanation that has been suggested previously is that p120 GAP, which is abundant and active in $Nf1^{-/-}$ hematopoietic cells (27, 30), is able to downregulate Ras-GTP when it associates in a complex with the IL-3 receptor and other proteins, but not when Ras associates with the GM-CSF receptor (27, 30). Biochemical evidence that MAP kinase is hyperactivated in c-kit$^+$ $Nf1^{-/-}$ cells in response to IL-3, or SCF (Fig. 5), and cell culture data showing that SCF alone and in combination with IL-3 or GM-CSF induce a hypersensitive pattern of in vitro growth in $Nf1^{-/-}$ cells (Fig. 2), suggest that aberrant intracellular signaling occurs in response to IL-3, GM-CSF, and SCF. However, the biochemical abnormality is not translated to hypersensitive colony formation in response to IL-3 only. It is possible that SCF and GM-CSF activate downstream signaling molecules important for terminal differentiation that are not induced by IL-3. For example, focal adhesion kinase is a recently identified kinase involved in the terminal differentiation of myeloid cells that is activated in response to GM-CSF (58) and SCF (59) but not IL-3. *Nf1*-deficient hematopoietic cells and cell lines should prove useful for further characterizing signaling pathways that are differentially activated and regulated in response to GM-CSF and IL-3. Our studies of purified Sca1$^+$lin$^{-/dim}$ cells suggest that the hypersensitive pattern of colony formation is intrinsic to the progenitors and is not dependent on accessory cells as suggested by some (31–33), but not all (21), previous studies in JCML cells.

Cultures established from primary d13.5 $Nf1^{-/-}$ fetal liver cells also demonstrated an increase in the numbers of primitive progenitor (HPP-CFC) cells in the fetal liver. In fact the expansion in this compartment was greater than was noted for more differentiated progenitors (LPP-CFC) scored in simultaneous experiments (our unpublished results). As the HPP-CFC and LPP-CFC assays were performed in the presence of saturating concentrations of hematopoietic growth factors, our data indicate that inactivation of *Nf1* is not only associated with a leftward shift in the dose–response curves for colony formation in response to exogenous growth factors but also with an absolute increase in the numbers of clonogenic progenitors in vivo. Preliminary data indicate that this increase in progenitors is associated with a relative resistance of $Nf1^{-/-}$ cells to apoptosis upon withdrawal of hematopoietic growth factors (Zhang, Y.-Y., and D.W. Clapp, unpublished results).

Biochemical data from c-kit positive hematopoietic cells demonstrating constitutive activation of MAP kinase signaling and a prolonged hyperactivation of MAP kinase activity in response to stimulation with cytokines suggests that deregulation of the Ras/MAP kinase pathway accounts for the perturbations of in vitro colony growth. Indeed, reducing the MAP kinase activity with an inhibitor of MEK was associated with a profound, specific reduction in colony formation from $Nf1^{-/-}$ c-kit$^+$ cells. The elevated and sustained levels of MAP kinase that we measured in response to SCF, IL-3, or GM-CSF in highly progenitor-enriched primary cells are consistent with the previous observation of a sustained elevation of Ras-GTP levels after GM-CSF stimulation in *Myb* immortalized $Nf1^{-/-}$ fetal hematopoietic cell lines (27). Similarly, primary bone marrow cells from children with NF1 and leukemia showed an elevated percentage of Ras-GTP (30). Although the biochemical data presented here, the predilection to myeloid leukemia seen in children with NF1 and in $Nf1^{+/-}$ mice, and the myeloid disorder that invariably develops in the recipients of $Nf1^{-/-}$ fetal liver cells provide compelling evidence that neurofibromin is essential to regulate Ras output in myeloid lineage cells, future studies are indicated to further delineate how neurofibromin modulates Ras in response to cytokines such as SCF. In particular, p120 GAP accounts for most of the in vivo and in vitro GTPase activating protein activity in bone marrow cells (30, 60, 61). It is possible that p120 GAP is sequestered away from growth factor receptor–Ras complexes in activated myeloid cells or is inactivated by a more direct mechanism. In this respect, the recent cloning of the gene encoding p62^{Dok} (Dok) is of interest as this protein is phosphorylated in leukemic cells that carry the *BCR-ABL* translocation and phosphorylated

Dok selectively associates with p120 GAP through Src homology 2 (SH2) domains. Dok is rapidly phosphorylated in hematopoietic cells in response to SCF (62). If the binding of phosphorylated $p62^{Dok}$ inhibits the function of p120 GAP, cells that have been stimulated with SCF may require neurofibromin to properly regulate Ras-GTP (neurofibromin does not contain SH2 domains [for review see references 4, 63, 64], and is unlikely to interact with Dok). Additional biochemical data are required to examine if SCF-induced phosphorylation of $p62^{Dok}$ affects the function of p120 GAP in normal and *Nf1*-deficient myeloid lineage cells.

In summary, we have shown that the loss of *Nf1* results in constitutive activation of Ras signaling in primitive and lineage-restricted hematopoietic progenitors. This is associated with hyperresponsiveness to multiple growth factors in biochemical and progenitor colony-forming assays, as well as in expansion of myeloid compartments in $Nf1^{-/-}$embryos and in adult recipients reconstituted with $Nf1^{-/-}$ fetal liver cells. Taken together, these data strongly suggest that the size and growth factor sensitivity of cells within these multiple hematopoietic compartments are regulated by neurofibromin through its ability to effect the activation state of the Ras/MAP kinase cascade. These data have implications for the use of GM-CSF antagonists to treat JCML. On the one hand, our data showing that inactivation of *Nf1* results in aberrant signaling in response to other growth factors provide evidence that GM-CSF–specific inhibitors might not show efficacy. However, mice that overexpress GM-CSF develop a JCML-like disorder (65), and in one recent study xenograft mice transplanted with JCML cells and treated with antagonists to GM-CSF had a significant reduction in JCML cells as compared with control mice treated with an isoimmune nonspecific antibody (66). Thus, it may be that the loss of *Nf1* results in aberrant Ras activation in multiple myeloid compartments, the importance of GM-CSF on terminal differentiation may be pivotal to acquisition of the clinical disease, and it remains possible that the clinical disorder will be abrogated by blocking the GM-CSF receptor. *Nf1* mice provide a valuable model system to test basic aspects of myeloid growth control and to evaluate novel therapeutics directed against hyperactive Ras.

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