The transcription factor Gfi1 regulates G-CSF signaling and neutrophil development through the Ras activator RasGRP1

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The transcription factor growth factor independence 1 (Gfi1) and the growth factor granulocyte colony-stimulating factor (G-CSF) are individually essential for neutrophil differentiation from myeloid progenitors. Here, we provide evidence that the functions of Gfi1 and G-CSF are linked in the regulation of granulopoiesis. We report that Gfi1 promotes the expression of Ras guanine nucleotide releasing protein 1 (RasGRP1), an exchange factor that activates Ras, and that RasGRP1 is required for G-CSF signaling through the Ras/mitogen-activated protein/extracellular signal-regulated kinase (MEK/Erk) pathway. Gfi1-null mice have reduced levels of RasGRP1 mRNA and protein in thymus, spleen, and bone marrow, and Gfi1 transcription in myeloid cells promotes RasGRP1 expression. When stimulated with G-CSF, Gfi1-null myeloid cells are selectively defective at activating Erk1/2, but not signal transducer and activator of transcription 1 (STAT1) or STAT3, and fail to differentiate into neutrophils. Expression of RasGRP1 in Gfi1-deficient cells rescues Erk1/2 activation by G-CSF and allows neutrophil maturation by G-CSF. These results uncover a previously unknown function of Gfi1 as a regulator of RasGRP1 and link Gfi1 transcriptional control to G-CSF signaling and regulation of granulopoiesis. (Blood. 2010; 115(19):3970-3979)

Introduction

Lineage-restricted transcription factors regulate differentiation of hematopoietic progenitors by promoting lineage-specific transcriptional programs and simultaneously repressing the transcriptional profile of alternative lineages. PU.1, CCAAT enhancing-binding protein (C/EBP) α and β are transcription factors that regulate neutrophil differentiation from progenitors.1 Deletion of either gene leads to the absence of neutrophils in conjunction with variable alterations of eosinophils, lymphocytes, and monocytes.

Growth factor independence 1 (Gfi1) is a zinc-finger transcription factor first identified as a gene frequently targeted for proviral integration contributing interleukin-2 growth independence in a rat lymphoma cell line and promoting T-cell lymphoma development.2–4 Gfi1 is expressed in thymus, spleen, testis, and the hematopoietic system.2–4 In hematopoietic stem cells, Gfi1 maintains quiescence.5,7 In myeloid cells, Gfi1 promotes differentiation.5,8,9 Gfi1-null mice lack normal neutrophils and accumulate a population of atypical Gr1+/CD11b+ cells that share characteristics of neutrophils and macrophages and can mature into macrophages but not neutrophils.5,8 The mice are small, die prematurely of bacterial infections, and have reduced T- and B-cell differentiation.5,8,10 Rare cases of severe congenital neutropenia have been linked to heterozygous Gfi1 mutations, which can act as dominant negative.9,11 The patients resemble Gfi1-null mice in displaying neutropenia, abnormal circulating myeloid precursors, and reduced B and T lymphocytes.9

Previous studies have concluded that Gfi1 regulates myeloid cell maturation by transcriptional repression of target genes, including suppressor of cytokine signaling 3 (SOCS3),12 neutrophil elastase,13 colony-stimulating factor1 (CSF1)/CSF1 receptor,11 and the microRNAs miR-21 and miR-196b.14 Derepression of these genes explains important aspects of defective neutrophil differentiation in Gfi1-null mice and patients with Gfi1 mutations. Gfi1 can bind consensus DNA target sequences by a C-terminal zinc-finger domain and repress transcription by its N-terminal SNAG domain,15 in part by recruiting corepressors.16–19 Besides acting as a transcriptional repressor, Gfi1 interacts with protein inhibitor of activated signal transducer and activator of transcription (STAT), which can bind to and inhibit STAT3 signaling.20 As a result, Gfi1 can relieve STAT3 from protein inhibitor of activated STAT3–induced inhibition, resulting in enhanced STAT3 signaling. Because STAT3s are activated by a variety of cytokines and growth factors, we hypothesized that Gfi1 could play a role as a modulator of cytokine and growth factor responses.

We focused on granulocyte (G)–CSF and its unique receptor (G-CSFR), which are critical to the generation of neutrophils from granulocyte/monocyte precursors and their release to the peripheral circulation. Homozygous deletion of G-CSFR or G-CSF causes severe neutropenia in mice.21,22 and heterozygous mutations of G-CSFR have been linked to sporadic cases of severe congenital neutropenia.23 As both Gfi1 transcriptional regulation and G-CSF/G-CSFR signaling are critical to neutrophil maturation, we have investigated whether their activities might be linked. We find that Gfi1 functions as a regulator of G-CSF/G-CSFR signaling in myeloid cells by promoting expression of RasGRP1 (Ras guanine nucleotide releasing protein 1), which is shown to be a critical modulator of G-CSF/G-CSFR–induced Ras activation.
Methods

Mice and cell isolation
Germline Gfi1−/− mice were bred and housed in the animal facilities at the National Cancer Institute. Genotyping was as described. All animal studies were approved by the National Cancer Institute Bethesda Animal Care and Use Committee and were carried out per protocol with mice to 8 weeks of age. G-CSF–induced cell mobilization experiments were described. Bone marrow, thymic, and splenic cell suspensions were prepared from isolated organs by standard techniques. Peripheral blood cell counts and differentials were carried out by the National Institutes of Health Clinical Center with the use of an ADVIA 120 hematology system (Bayer).

Cell culture
Bone marrow mononuclear cells (MNCs) were obtained by flushing femurs and tibias, and Lin− cell bone marrow cell populations were derived with theMouse Lineage Cell Depletion Kit (Miltenyi Biotec; 130-090-858) as described by the manufacturer. For signaling experiments, cells (1-2 × 10^6/mL) were incubated (0-180 minutes) in Iscove Dulbecco modification of Eagle medium (DMEM) with 5% FBS and 1% antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C in a 5% CO2 humidified atmosphere. For proliferation experiments, cells were cultured (1 × 10^6 cells/well in 96-well plate) for 3 days in Iscove modified Dulbecco medium with 10% FBS with or without G-CSF (25 ng/mL) or interleukin-3 (IL-3; 10 ng/mL). For differentiation experiments in liquid culture, cells were incubated (5 × 10^3 to 1 × 10^4) in Iscove DMEM with 10% FBS with or without G-CSF (25 ng/mL) with or without puromycin (2.5 mg/mL). Colony assays were performed with methocult M3234 (StemCell Technologies). Bone marrow cells (5 × 10^6 cultures supplemented with 10% FBS; IL-3; PropoTech), 10 ng/mL IL-3 (R&D Systems), and 50 ng/mL stem cell factor (SCF; R&D Systems) or 4 × 10^6 cells (cultures supplemented with 25 ng/mL G-CSF) were plated into 35-mm² petri dishes and incubated for 10 to 14 days. The murine 32Dcl3 cell line (32D; a gift of Dr A. Friedman, John Hopkins University) was maintained in DMEM supplemented with 10% FBS and 1 ng/mL murine IL-3. For signaling experiments, 32D cells were washed and preincubated in DMEM supplemented with 10% FBS medium for 4 hours; cells (1-2 × 10^6/mL) were harvested, washed, and activated with 25 ng/mL G-CSF or 10 ng/mL IL-3.

Cell proliferation
Proliferation was measured by 3H-thymidine deoxyribose incorporation and activated with 25 ng/mL G-CSF or 10 ng/mL IL-3. For proliferation experiments, cells were cultured (1 × 10^6 cells/well in 96-well plate) for 3 days in Iscove modified Dulbecco medium with 10% FBS with or without G-CSF (25 ng/mL) or interleukin-3 (IL-3; 10 ng/mL). For differentiation experiments in liquid culture, cells were incubated (5 × 10^3 to 1 × 10^4) in Iscove DMEM with 10% FBS with or without G-CSF (25 ng/mL) with or without puromycin (2.5 mg/mL). Colony assays were performed with methocult M3234 (StemCell Technologies). Bone marrow cells (5 × 10^6 cultures supplemented with 10% FBS; IL-3; PropoTech), 10 ng/mL IL-3 (R&D Systems), and 50 ng/mL stem cell factor (SCF; R&D Systems) or 4 × 10^6 cells (cultures supplemented with 25 ng/mL G-CSF) were plated into 35-mm² petri dishes and incubated for 10 to 14 days. The murine 32Dcl3 cell line (32D; a gift of Dr A. Friedman, John Hopkins University) was maintained in DMEM supplemented with 10% FBS and 1 ng/mL murine IL-3. For signaling experiments, 32D cells were washed and preincubated in DMEM supplemented with 10% FBS medium for 4 hours; cells (1-2 × 10^6/mL) were harvested, washed, and activated with 25 ng/mL G-CSF or 10 ng/mL IL-3.

Cell sorting, flow cytometry, and immunocytochemistry
Green fluorescent protein (GFP)–expressing cells and cells surface-stained with phycoerythrin (PE) rat anti–mouse Gfi1 antibodies (BD PharMingen) were sorted with a FACS Vantage SE (BD Biosciences). For analysis, cells were stained with specific antibodies: allophycocyanin (APC) rat anti–mouse CD45/R2B220 APC rat anti–mouse CD11b (R: Red) PE-conjugated rat anti–mouse CD4, fluorescein isothiocyanate (FITC) and PE conjugated rat anti–mouse CD117 (cKit, FITC rat anti–mouse Ly-6A/E (Sca-1), FITC rat anti–mouse CD58a, PE/cyanine 5 (Cy5) rat anti–mouse CD45, PE/Cy5 anti–mouse CD45, PE/Cy5 anti–mouse CD48; PE/Cy5 anti–mouse CD150, PE rat anti–mouse CD16/CD32, and relevant isotype control antibodies (all from BD PharMingen); PE rat anti–mouse CD34/CD150, PE anti–mouse CD242.2 (BioLegend); biotin-labeled Lin selection cocktail (StemCell Technologies); R:PE rat anti–mouse neutrophils RPE (clone 7/4) and FITC rat anti–mouse F4/80 antigen (AbD Serotec); rabbit anti–phospho extracellular signal-regulated kinase 1/2 (Erk1/2) and total Erk1/2 monoclonal antibodies (Cell Signaling Technology) and PE-labeled goat anti–rabbit antibodies (Invitrogen); RasGRF1 monoclonal antibody m199 (Santa Cruz Biotechnology) or control mouse immunoglobulin G1 (IgG1; Invitrogen) followed by Alexa Fluor 488 goat anti–mouse IgG1 (Invitrogen). Surface G-CSFR was detected with PE-labeled G-CSF (R&D Systems) cross-linked with Bic(sulfosuccinimidyl)suberate (Pierce, Thermo Scientific). Staining for phospho and total Erk1/2 was carried out after fixation (10 minutes at room temperature in Iscove medium with 2% paraformaldehyde) and permeabilization in (60 minutes at 4°C in 90% methanol) with the use of specific primary antibodies followed by PE-labeled anti–rabbit secondary antibodies (BD PharMingen). Staining for RasGRF1 was carried out after cell fixation and permeabilization (Fix and Perm solution; BD Pharmingen) with the use of RasGRF1 monoclonal antibody m199 (Santa Cruz Biotechnology) or control mouse IgG1 (Invitrogen) followed by Alexa Fluor 488 goat anti–mouse IgG1 (Invitrogen). For double-staining cells were first surface-stained and then fixed/permeabilized for RasGRF1 staining. Results were analyzed with CellQuest software (BD Biosciences) after acquisition of data from 106 cells. Cytospin preparations were immunostained for intracellular RasGRF1 as described for flow cytometry; nuclei were stained with DAPI (4,6 diamidino-2-phenyindole); cell morphology was evaluated by Giemsa staining. Fluorescence and bright-field images were acquired through a Nikon Eclipse E600 microscope equipped with Plan Apo 40×/0.95 DIC M, 60×/1.40 oil differential interference contrast (DIC) H, and 100×/1.40 oil DIC H lenses, and photographed with a digital camera (Retiga 1300; QImaging). Images obtained with IPLab for Windows software (Scanalytics) were imported into Adobe Photoshop.

Retroviral constructs, infection, and small interfering RNA
Gfi1-GFP-RV and GFP-RV26 and pBabe-RasGRF1 or pBabe27 were previously constructed. Retroviruses were packaged in the Phoenix packaging cell line, as described.28 Two-day culture supernatants were filtered, supplemented with Polybrene (4 mg/mL; final concentration; Sigma-Aldrich) and used for infection. Two days after infection with Gfi1-GFP-RV and GFP-RV retroviruses, GFP-expressing 32D cells were sorted; only cell populations at least 80% GFP+ were used for experiments. Two days after infection with pBabe-RasGRF1 or pBabe retroviruses, puromycin (1 mg/mL) was added to 32D cells for selection. For infection of primary mouse bone marrow cells, a similar method was applied except for the use of RetroNectin-coated plates (Takara) and cell preculture (18-72 hour) in culture medium supplemented with IL-3 (10 ng/mL), cKitl (25 ng/mL), SCF (25 ng/mL), and granulocyte-macrophage-CSF (5 ng/mL). Two days after retroviral infection, the GFP+ and GFP− cells were sorted, and the cells used for further experiments. RasGRF1 was silenced with small interfering RNAs (siRNAs) for mouse RasGRF1 (Ambion, Applied Biosystems); rino-free siRNA (Dharmacon) was used as control. siRNA was transfected into 32D cells with the Amaxa nucleofector system (Amaxa Biosystems) optimized for 32D cells (E-32), with Cell Line Nucleofector Solution V. Transfection efficiency was assessed by GFP positivity.

RNA isolation and semiquantitative and real-time polymerase chain reactions
We isolated total RNA with TRIZol reagent (Invitrogen). Semiquantitative polymerase chain reaction (PCR) was performed as described,25 using primers for amplification of mouse Gfi1,26 mouse RasGRF1 (5′-GGACCACGGTGATAGTGCTT-3′; 5′-TGTCGACACTTGCAGCTT-3′), and glyceraldehyde phosphate dehydrogenase.38 Real-time PCR was performed with the use of Assay-on-Demand TaqMan probes (Applied Biosystems) for mouse RasGRF1, Gfi1, G-CSFR, and glyceraldehyde phosphate dehydrogenase; rat Gfi1 and CSF1.

Immunoprecipitation and Western blotting
RasGRF1 was immunoprecipitated with rabbit anti–RasGRF1 antibody (H120; Santa Cruz Biotechnology) from cell extracts prepared in NP-40 lysis buffer supplemented with protease inhibitor cocktail set III (Calbiochem) supplemented with 50 mM NaF and phenylmethyl sulfonyl fluoride. Precipitates were resolved through 8% Tris Glycine Gels (Invitrogen) and immunoblotted with mouse monoclonal anti–RasGRF1 antibody m199 or rabbit anti–RasGRF1 (H120).29 Active Ras was immunoprecipitated with
glutathione-S-transferase–Raf1 (residues 1-149; Upstate) following the manufacturer’s instructions; precipitates were resolved through 10% NuPage Bis Tris gels (Invitrogen) and immunoblotted with anti-Ras antibody (05-516; Upstate). Protein extracts for phosphorylated proteins prepared in sodium dodecyl sulfate lysis buffer with protease inhibitor cocktail set III (Calbiochem), 50mM NaF; and 1mM sodium orthovanadate were resolved in 4% to 12% or 10% NuPAGE Bis Tris or 10% to 20% Tricine gels (Invitrogen), and nitrocellulose membranes were immunoblotted with the following specific antibodies: phospho-p44/42 mitogen-activated protein (MAP) kinase (Tyr202/Tyr204), total p44/p42, phospho-MAPK/Erk1/2 (MEK1/2) (Ser217/221), phospho–Stat-3 (Tyr705), total Stat-3 (BD Biosciences), phospho–Stat-1(Tyr701), phospho–Stat-5(Tyr694), phospho-Akt (Ser473), total Akt, phospho-Erk5 (Thr218/Tyr220), phospho-p38 MAP kinase (MAPK) (Thr180/Tyr182), phospho-Src (Tyr527 and Tyr416), non–phospho-Src (Tyr527), phospho-MAPK phosphatase 1 (MKP1) (Ser359,125E2), phospho–KSR1 (Ser392), phospho–c-Raf (Ser259), SOCS3 (2923) (all from Cell Signaling Technology); SOCS3 (M-20 and H-103; Santa Cruz Biotechnology), SOCS3 (Zymed Laboratories), MKP1 (C-19; Santa Cruz Biotechnology), and phospho-c-Jun (Ser73; Upstate).

**Statistical analysis**

Group differences were evaluated by 2-tailed Student t test. P values less than .05 were considered significant.

**Results**

**Altered G-CSF signaling in Gfi1-deficient hematopoietic cells**

Consistent with previous studies, Gfi1-null mice from our colony have a reduction in circulating granulocytes (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article) and in the mobilization response to G-CSF administration in vivo (supplemental Table 2), deficiencies that could derive from defective G-CSF responses.

G-CSF signals through the G-CSFR, which is expressed exclusively from myeloid-restricted progenitor cells to mature neutrophils.30 We found G-CSFR expression levels to be reduced somewhat lower in Gfi1−/− bone marrow MNCs, whereas STAT1, STAT3, and p38MAPK phosphorylation was similar. STAT5 phosphorylation was also found reproducibly reduced in Gfi1−/− bone marrow MNCs, whereas STAT1, STAT3, and p38MAPK phosphorylation was similar. STAT5 phosphorylation was also somewhat lower in Gfi1−/− bone marrow MNCs than in controls. We tested whether expression of Gfi1 in Gfi1−/− bone marrow MNCs could reconstitute Erk1/2 activation by G-CSF.

Using a retroviral vector for expression of GFP-Gfi1 and sorting the GFP+ and GFP− cell populations, we found that expression of Gfi1 in Gfi1−/− bone marrow MNCs reconstituted Erk1/2
RasGRP1 mRNA is substantially more abundant than in bone
induced) that were not attributable to a Ras protein deficiency in the
Gfi1 allowing Ras binding to cellular GTP.33 By real-time PCR, we
Ras activity by catalyzing the release of guanosine diphosphate,
addition, consistent with active Ras serving as an inducer of the
Raf/MEK/Erk pathway, reduced Ras-GTP was accompanied by
responding to the Raf1 domain that binds to Ras–guanosine
triphosphate [GTP] detected significantly greater amounts of
lysates from Gfi1 mice predicted similar low levels in bone
marrow MNCs from Gfi1/+/+ and Gfi1−/− mice (not shown).
Phosphorylated (serine [Ser] 359) MKP1 and Akt (Ser473) were
also detected at similarly low levels in bone marrow MNCs from
Gfi1−/− and Gfi1+/+ mice, with or without G-CSF (not shown).

To explore whether there might be a global defect in Erk1/2
signaling, we stimulated bone marrow MNCs with IL-3, which
activates the Erk1/2 pathway through the IL-3 receptor, a receptor
that is functional in most nucleated hematopoietic cells.32 However,
bone marrow MNCs from Gfi1+/+, Gfi1+/−, and Gfi1−/− mice
similarly activated Erk1/2 phosphorylation with 10 ng/mL IL-3
(Figure 2A), which was a stronger activator of Erk1/2 than G-CSF
(Figure 2B), presumably reflective of the wider expression of
IL-3–responsive cells in bone marrow MNCs.

The defect in MEK1/2 and Erk1/2 phosphorylation after G-CSF
stimulation of bone marrow MNCs from Gfi1−/− mice suggested
that Ras activation might be impaired in these cells. Consistent
with this possibility, a pull-down assay of active Ras (using
agarose-bound glutathione-S-transferase–Raf1 residues 1-149, cor-
responding to the Raf1 domain that binds to Ras–guanosine
triphosphate [GTP]) detected significantly greater amounts of
active Ras (constitutive and G-CSF–induced) in bone marrow cell
lysates from Gfi1+/+ in comparison to Gfi1−/− mice (Figure 2C),
differences (both constitutive and G-CSF–induced) that were not attributable to a Ras protein deficiency in the
bone marrow cell lysates from Gfi1−/− mice (Figure 2C). In
addition, consistent with active Ras serving as an inducer of the
Raf/MEK/Erk pathway, reduced Ras-GTP was accompanied by
reduced phospho-Erk1/2 in Gfi1−/− cells (Figure 2C).

RasGRP1 deficiency in Gfi1-deficient mice
To identify genes whose expression might underlie the defective
Ras activation in Gfi1-null mice, we used microarray analysis to
compare patterns of gene expression in bone marrow MNCs from
Gfi1−/− and Gfi1+/+ mice. Among the genes whose expression
differed by at least 5-fold, we found that RasGRP1 mRNA was
approximately 8-fold less abundant in bone marrow MNCs from
Gfi1−/− mice than in Gfi1+/+ mice. RasGRP1s are Ras guanine
nucleotide-exchange factors (Ras GEFs) that positively regulate
Ras activity by catalyzing the release of guanosine diphosphate,
allowing Ras binding to cellular GTP.35 By real-time PCR, we
confirmed that levels of RasGRP1 mRNA were reduced in the bone
marrow MNCs of Gfi1−/− mice compared with Gfi1+/+ and
Gfi1+/− mice (Figure 3A). In the thymus and spleen, where
RasGRP1 mRNA is substantially more abundant than in bone
marrow, levels of RasGRP1 mRNA were also reduced in Gfi1−/−
mice compared with controls (Figure 3A). This difference in
RasGRP1 mRNA was confirmed at the protein level in the thymus
and spleen by immunoprecipitation/immunoblotting (Figure 3B).
Consistent with the low level of mRNA in bone marrow (Figure
3A), RasGRP1 protein was not clearly detected in bone marrow
cells even from wild-type mice (Figure 3B). Nonetheless,
RasGRP1 mRNA was consistently detected in bone marrow
MNCs from Gfi1−/− mice compared with Gfi1+/+ and Gfi1+/− mice.
Freshly obtained cells were incubated for 10 minutes in either medium only or with IL-3 (10 ng/mL). The results reflect
reprobing of a single membrane. The results are representative of 7 experiments. (B) Phospho-Erk1/2 activation by IL-3 or G-CSF in Gfi1−/− and Gfi1−/− bone marrow
MNCs evaluated by immunoblotting with specific antibodies. Cells were incubated for
10 minutes in medium only or with IL-3 (10 ng/mL) or G-CSF (25 ng/mL). The results
are representative of 5 experiments. (C) Active Ras was pulled down from bone
marrow cells lysates with agsarose-bound Raf-1 protein (residues 1-149 corresponding
to the binding domain for Ras-GTP) and immunoblotted with Ras-specific antibody.
Bone marrow MNCs from 2 Gfi1−/− and 2 Gfi1+/− mice were incubated in medium
only or with G-CSF (25 ng/mL) for 10 minutes; the cell lysates were either
immunoblotted directly or after Ras-GTP pull down. The membrane was reprobed
with specific antibodies to phospho-Erk1/2 or total Erk. The results are representative
of 3 experiments performed.

RasGRP1 mRNA was confirmed at the protein level in the thymus
and spleen by immunoprecipitation/immunoblotting (Figure 3B).
Consistent with the low level of mRNA in bone marrow (Figure
3A), RasGRP1 protein was not clearly detected in bone marrow
cells lysates even from wild-type mice (Figure 3B). Nonetheless,
RasGRP1 mRNA was consistently detected in bone marrow MNCs
from Gfi1+/+ and Gfi1−/− mice and to a significantly (P < .05)
lower degree in Gfi1−/− mice (Figure 3C).

By flow cytometry, we identified in the bone marrow MNCs of
control mice a small (0.5%-3.0%) population of cells with intracel-
ular RasGRP1, which was not found in the Gfi1-null mice (representative results in Figure 3D). These cells expressing
RasGRP1 displayed a high side scattering counter (SSC; Figure
3E) and low surface Gr1 expression (Figure 3F), characteristics
compatible with immature granulocytic precursors.34 By double
staining, the RasGRP1-expressing cells did not stain for the
neutrophil marker 7/4, the monocyte/macrophage marker CD11b,
the T-cell marker CD4, the pan-B cell marker CD45R/B220, or the
hematopoietic cell progenitor marker cKit; most of the cells
expressed the G-CSFR, confirming their probable myeloid lineage derivation (Figure 3G). Further analysis showed that the RasGRP1-expressing cells were found within the CMP (lin^−Sca^−c-Kit^−CD34^−CD16/32^mid^) and GMP (lin^−Sca^−c-Kit^−CD34^−CD16/32^hi^) cell populations (Figure 3H).

We sorted bone marrow MNCs that exhibited high SSC and low- or high-level Gr1 expression. Consistent with previous observations, forward scattering counter (FSC) and SSC of bone marrow MNCs from Gfi1^−/−^ mice differed from that of Gfi1^+/+^ mice in showing a reduction of cells with high SSC and low Gr1 expression.
intermediate forward scattering counter (Figure 4A). Consistent with their defective neutrophil maturation, Gfi1-null mice showed a reduction in the Gr1hi cell population and an expansion of Gr1lo cell population (Figure 4A right). Within the Gr1hi cell population from Gfi11+/+ mice, we identified cells that specifically stained brightly with RasGRP1 mAb (Figure 4B). However, no RasGRP1-staining cells were identified within the Gr1hi cell population from Gfi11−/− mice (Figure 4B). RasGRP1 staining was mostly confined to the plasma membrane/cytoplasm, and the cells that stained for RasGRP1 displayed the nuclear morphology of immature cells (Figure 4B-D). No RasGRP1-brightly positive cells were identified within the more mature myeloid cells, based on nuclear morphology (DAPI and Giemsa staining) and Gr1hi expression (Figure 4B-C).

**Gfi1 regulates RasGRP1 expression and signaling**

To test for the possibility that Gfi1 may induce RasGRP1 expression and that RasGRP1 deficiency may contribute to defective G-CSF signaling and function in Gfi11−/− mice, we stably transduced the murine myeloid 32D cells with rat Gfi1 or rat RasGRP1 retroviral vectors, because these cells do not constitutively express detectable levels of Gfi1 or RasGRP1 (Figure 5A). Forced expression of Gfi1 in these cells was accompanied by increased expression of endogenous RasGRP1 mRNA (Figure 5B-C), but not G-CSFR (not shown), and increased phosphorylation of Erk1/2 with G-CSF, but not IL-3 (Figure 5D). Conversely, silencing RasGRP1 in Gfi1-transduced 32D cells resulted in decreased phosphorylation of Erk1/2 with G-CSF, but not IL-3 (Figure 5E). In addition, overexpression of RasGRP1 in 32D cells resulted in increased phosphorylation of Erk1/2 on stimulation with G-CSF, but less clearly with IL-3. By contrast, RasGRP1 expression in 32D cells was accompanied by minimal change in G-CSF–induced activation of STAT3 and STAT5 (Figure 5F) and expression of G-CSFR (not shown).

G-CSF can stimulate Gfi1 expression in 32D cells under culture conditions that promote their terminal differentiation into neutrophils. We now found that G-CSF additionally stimulates expression of endogenous RasGRP1 in 32D cells after the induction of Gfi1 (Figure 5G). Thus, Gfi1 promotes expression of RasGRP1 in 32D cells, which specifically enhances Erk1/2 phosphorylation in response to G-CSF.

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**Figure 4. Characterization of RasGRP1-expressing cells in the bone marrow.** (A) FSC and SSC of bone marrow cells from a representative Gfi11+/+ and Gfi11−/− mouse showing the different distribution pattern (left). Distribution of bone marrow cells expressing high (hi), intermediate, and low (lo) levels of surface Gr1 showing a reduction of Gr1hi and an increase in Gr-1lo in a representative Gfi11−/− mouse compared with control Gfi11+/+ mouse. The percentage of cells within each gate is displayed (representative results from 5 experiments). (B) Cytospin preparations of sorted bone marrow Gr1hi and Gr1lo cell populations from a control Gfi11+/+ mouse showing that RasGRP1-stained cells have the nuclear morphology of immature myeloid cells and are confined to the Gr1lo cell populations. Mouse IgG1 isotype antibody was used as a negative control; DAPI shows nuclear morphology. RasGRP1 is undetectable in bone marrow Gr1lo cell populations from Gfi11−/− mice (representative results from 5 experiments). (C) Giemsa and DAPI staining of Gr1hi-sorted bone marrow populations after immunostaining for intracellular RasGRP1 showing cellular morphology. RasGRP1 is detected in Gr1hi bone marrow cell populations from a Gfi11+/+ mouse but not from a Gfi11−/− mouse. Representative images are shown. (D) Enlarged image from Giemsa stained Gr1lo-sorted bone marrow populations showing the morphology of cells expressing RasGRP1 from the control Gfi11+/+ mouse (top). The morphology of Gr1lo cell populations from the Gfi11−/− mouse that fail to display RasGRP1 immunostaining is shown (bottom) in representative images.
RasGRP1 promotes G-CSF–dependent neutrophil differentiation from hematopoietic progenitor cells

On withdrawal of IL-3 and the addition of G-CSF, 32D cells differentiate from myeloblasts into more mature granulocytic cells over a period of days. We examined whether forced RasGRP1 expression led to increased 32D cell differentiation with G-CSF. Staining with DAPI distinguishes the nuclei of myeloblasts from those of more mature myeloid cells, based on the absence of cavitation, lobulation, or fragmentation (Figure 6A). In kinetic experiments, we found that RasGRP1-transduced 32D cells differentiated more rapidly than control cells (Figure 6A). Gfi1-transduced 32D cells showed differentiation kinetics similar to those of RasGRP1-transduced cells (Figure 6A). After a 4-day incubation with G-CSF, granulocytic differentiation was consistently greater in Gfi1– or RasGRP1-transduced 32D cells than in controls (P < .05; Figure 6B). Thus, functionally, Gfi1 and RasGRP1 similarly support G-CSF–induced granulocytic differentiation of 32D cells.

Because the transient expression of Gfi1 in Gfi1-null granulocytic progenitors partially corrected the defect in neutrophil differentiation and promoted generation of mature neutrophils,5 we tested the effects of RasGRP1 expression in these assays. With the use of a retroviral vector for expression of RasGRP1 and puromycin selection, we increased relative RasGRP1 expression in Gfi1−/− bone marrow MNCs by 3- to 4-fold (Figure 6C). When transduced with RasGRP1 and stimulated with G-CSF, Gfi1−/− bone marrow cells activated Erk1/2, albeit to a somewhat lower degree than control Gfi1+/+ cells, whereas Gfi1−/− bone marrow cells transduced with the empty retroviral vector essentially failed to do so (Figure 6D).

Colony assays in semisolid medium supplemented with IL-3, IL-6, and SCF showed an increase in progenitors responsive to these cytokines from Gfi1−/− bone marrow cells compared with Gfi1+/+ control, a difference that persisted when the cells were transduced with RasGRP1 (Figure 6E). Most (> 90%) of the colonies from the Gfi1−/− control and RasGRP1-transduced cells were megakaryocyte colony-forming unit (CFU-M), and the remainder (≤ 10%) were granulocyte-macrophage CFU (CFU-GM). By contrast, the colonies from the Gfi1+/+ precursors were 50% granulocyte CFU, 39% CFU-M, and the remaining 11% were CFU-GM. Colony assays in semisolid medium supplemented with G-CSF also showed an increase in progenitors responsive to this cytokine from Gfi1-null bone marrow cells compared with Gfi1+/+ control (Figure 6F). Virtually all the colonies from Gfi1+/+ precursors were granulocyte CFU, whereas virtually all the colonies from Gfi1−/− vector-transduced precursors were CFU-M. Instead, 30% to 50% of colonies from Gfi1−/− cells transduced with RasGRP1 were CFU-G, and the remainder were CFU-M. Importantly, microscopic analysis of G-CSF–induced colonies (cytospin preparations) from Gfi1-null cells transduced with RasGRP1 showed the presence of clusters of cells at different stages of granulocyte differentiation, including mature neutrophils, which were generally missing from control Gfi1-null bone marrow cells (not shown). To confirm that introduction of RasGRP1 in Gfi1−/− bone marrow cells promoted neutrophil differentiation, we used liquid cultures supplemented with G-CSF. After a 4-day incubation, we counted mature neutrophils in cytospin preparations. In 3 experiments, we found that expression of RasGRP1 in Gfi1−/− bone marrow cells promoted substantial neutrophil differentiation in the presence of G-CSF (P < .05 vector vs RasGRP1), which was largely missing from control cultures (Figure 6G). Thus, introduction of RasGRP1 correctly, at least in part, the defect in neutrophil differentiation resulting from the loss of Gfi1.

Discussion

The interplay between transcription factors and cytokines/growth factors that regulate differentiation of myeloid cells from hematopoietic precursors remains largely undefined. Individually, the transcription factor Gfi1 and the cytokine G-CSF are critical
regulators of neutrophil maturation from granulocyte/monocyte progenitors. Here, we show that the transcription factor Gfi1 regulates G-CSF signaling through the Ras/MEK/Erk pathway, by stimulating the expression of RasGRP1, a critical regulator of Ras activation. Thus, the current results uncover a previously unknown link between Gfi1 transcriptional control and G-CSF signaling in the regulation of granulopoiesis.

RasGRP1 is a GEF that activates the small GTPase Ras. Levels of active Ras are regulated by the equilibrium between inactive guanosine diphosphate–bound Ras and active GTP-bound Ras. This equilibrium is regulated by Ras GEFs, which activate Ras, and Ras GTPase-activating proteins (GAPs), which inactivate Ras. RasGRP1 has a limited tissue distribution, being detected predominantly in thymocytes, T cells, and various T-cell lines and...
to a lower degree in B cells. Interestingly, RasGRP1 mRNA was detected in the promyelocytic leukemia cell line HL60 (Gene Atlas U133A, gcra; 205590) and bone marrow from patients with acute myelogenous leukemia. Here, we show that RasGRP1 is expressed in the normal bone marrow but not in the bone marrow of Gfi1-null mice. We detect RasGRP1 in 1% to 3% of normal bone marrow but not in the bone marrow of Gfi1-null mice. We detect RasGRP1 in 1% to 3% of normal bone marrow MSCs; these RasGRP1+ cells display an immature myeloid cell phenotype, including myeloblasts and promyelocytes. Because promyelocytes comprise 3.7% (± 0.5%) of bone marrow MNCs from 4- to 6-week-old C57BL/6 mice, a considerable proportion of myeloid cells at this stage may express RasGRP1. The failure to detect RasGRP1 in Gfi1-null bone marrow cannot be attributed to a putative reduction of granulocyte progenitors, because such cells are increased in Gfi1-null bone marrow. Because we observed that RasGRP1 is expressed at abnormally low levels in thymus and spleen of Gfi1-null mice, it is possible that RasGRP1 deficiency may also contribute to the T- and B-cell deficiencies in Gfi1-deficient mice and patients.

An important question is how RasGRP1 is biochemically linked to G-CSFR. In lymphocytes, immune receptor signaling leads to phospholipid hydrolysis. The resulting diacylglycerol positively regulates RasGRP1 both directly, through membrane recruitment, and indirectly, through protein kinase C–mediated phosphorylation. However, evidence that G-CSFR couples to phospholipid hydrolysis is lacking. G-CSFR signaling to Ras is thought to involve phosphorylation of one or more C-terminal tyrosines, which are presumed to serve as docking sites for adaptor proteins Grb2 and SHC, thereby facilitating membrane recruitment of the Ras GEF Sos. This proximal region may signal directly or indirectly, through protein kinases or adaptor proteins, to RasGRP1. The interplay of the 2 Ras GEFs in myeloid cells might allow a variety of intracellular and kinetic patterns of Ras-Erk signaling, resulting in distinct cellular outputs.

Another question is how RasGRP1 deficiency alone could account for defective Erk1/2 activation by G-CSF. It is possible that RasGRP1 is more widely expressed among G-CSF–responsive cell populations than we conservatively estimated, based on brightly positive immunofluorescence staining. It is also possible that other factors, besides RasGRP1, contribute to Erk activation by G-CSF. Consistent with the latter possibility, transduction of Gfi1 or RasGRP1 in Gfi1-null bone marrow cells only partially rescued Erk activation by G-CSF.

G-CSF promotes growth and differentiation in responsive cells, but the signaling pathways that mediate these distinct cytokine functions remain incompletely defined and are probably complex. In Gfi1-null myeloid cells, where little or no Erk1/2 is activated by G-CSF, we found that G-CSF promotes exaggerated cell proliferation (shown here) and colony formation, but minimal neutrophil differentiation. At least partial neutrophil maturation by G-CSF occurred with reconstitution of Ras/MEK/Erk signaling, supporting the notion that the Ras/MEK/Erk pathway contributes to G-CSF–induced neutrophil differentiation. A previous study with 32D cells bearing a mutant G-CSFR suggested a potential role for Ras activation in maintaining G-CSF–induced proliferation rather than differentiation. It is possible that different Ras activators may lead to distinct downstream pathways and that RasGRP1 activates Ras in unique ways, as it was proposed in the case of T cells. The Raf-1/MAK pathway is one of several pathways regulated by active Ras, which can bind to several effector proteins, including phosphatidylinositol 3 kinase and the small GTPase Ral. It is also possible that proliferative and differentiation responses in myeloid cells share the same signaling pathway, but the duration of Erk activation differs. In PC12 cells, short-lived Ras-Erk activation leads to proliferation, whereas persistent activation of Ras-Erk leads to differentiation.

Previously, the monopoietic cytokine CSF-1 (colony stimulating factor-1/M-CSF), the transcription factor PU.1, and the microRNAs miR21 and miR-196b were reported to contribute to defective myelopoiesis in Gfi1-null mice. It is not surprising that defective granulopoiesis in the context of Gfi1 deficiency is multifactorial. Consistent with this possibility, granulopoiesis from Gfi1-null precursors was only partially reconstituted by neutralization of CSF-1, heterozygosity at the PU.1 locus, silencing miR21 and miR-196b, and transduction of RasGRP1, shown here.

Thus far, transcriptional repression of target genes has been identified as a principal mechanism of Gfi1 function as a regulator of granulopoiesis. This has contributed to models in which commitment to granulopoiesis is a default pathway. Here, we find evidence that Gfi1 can stimulate the expression of RasGRP1 and induce granulopoiesis G-CSF/G-CSFR differentiation, but the underlying mechanisms need investigation. It is possible that Gfi1 could function as a transcriptional activator of RasGRP1 expression. However, because we did not find conserved Gfi1 binding sequences in the putative promoter region of RasGRP1, Gfi1 may indirectly modulate RasGRP1 expression by repressing negative regulators. Other transcription factors can both activate and repress transcription of target genes. Myc, for example, activates transcription directly when bound to its DNA consensus sequence, but it represses transcription when tethered to target sequences by Miz1 or other cofactors. Besides expanding the spectrum of Gfi function as a regulator of hematopoiesis, the current observations will probably lead to important insights in myeloid cell development, maturation, and transformation that will inform therapies aimed at manipulating myelopoiesis.

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References

The transcription factor Gfi1 regulates G-CSF signaling and neutrophil development through the Ras activator RasGRP1

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