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G-CSF Induces Stabilization of ETS Protein Fli-1 during Myeloid Cell Development

PATRICIA MORA-GARCIA, JOLYN WEI, AND KATHLEEN M. SAKAMOTO

Department of Pediatrics [P.M.-G., J.W., K.M.S.], Division of Hematology-Oncology, Gwynne Hazen Cherry Memorial Laboratories, and Mattel Children's Hospital at UCLA, Los Angeles, California, 90095, Department of Pathology and Laboratory Medicine [K.M.S.], David Geffen School of Medicine and Jonsson Comprehensive Cancer Center, Molecular Biology Institute, UCLA, Los Angeles, California, 90095, Division of Biology [K.M.S.], California Institute of Technology, Pasadena, California, 91125

ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is a growth factor that regulates the production and function of neutrophils. G-CSF has been used to treat neutropenia in neonates, pediatric cancer patients, and patients undergoing stem cell transplantation. The regulation of transcription factors mediating G-CSF activity has not been well characterized. The goal of this study was to examine the regulation of the ETS binding protein, Friend leukemia integration site 1 (Fli-1), in myeloid cells treated with G-CSF. Fli-1 has oncogenic properties in humans and mice, and plays a role in vascular and hematopoietic cell development. We previously reported that Fli-1 and the serum response factor bind at adjacent sites within the serum response element-1 of the early growth response gene-1 promoter in the murine myeloid leukemic cell line, NFS60. We also identified that Fli-1 DNA binding increased in G-CSF–treated cells compared with untreated cells. To determine whether the change in binding activity is due to increased Fli-1 transcription or protein stability, we examined endogenous Fli-1 expression in G-CSF–treated or –untreated NFS60 cells. Our results demonstrated that levels of Fli-1 protein, but not RNA, were higher in extracts from cells treated with G-CSF. The increase in Fli-1 protein was also dependent on protein synthesis. Finally, we showed that the half-life of Fli-1 is prolonged in G-CSF–treated cells compared with control-treated cells. These results suggest that G-CSF induces stabilization of Fli-1 protein in myeloid cells, thus proposing a novel mechanism by which hematopoietic growth factors regulate transcription factors. (***Pediatr Res*** **57: 63–66, 2005)**

Abbreviations

egr-1, early growth response gene-1
Fli-1, Friend leukemia integration site 1
G-CSF, granulocyte colony-stimulating factor
SRF, serum response factor

G-CSF is a cytokine that stimulates the proliferation and maturation of myeloid precursors and enhances the function of mature granulocytes. G-CSF regulates both basal hematopoiesis and increases the production and release of neutrophils from the marrow in response to infection (1). This growth factor is used to ameliorate the complications of neutropenia in neonates, children with congenital severe neutropenias, and children with neutropenias associated with cytotoxic cancer therapy, stem cell transplantation, and human immunodeficiency virus infections (2–6). Although there have been extensive studies to define the signaling pathways mediating the biologic activities of G-CSF, little is known about the regulation of transcription factors that induce these effects in myeloid cells.

Recent evidence suggests that STATs (signal transducers and activators of transcription) and PU.1 play a major role in the fate of cells during myeloid cell differentiation (7–10). We propose that the ETS transcription factor Fli-1 is regulated by G-CSF signaling during myelopoiesis. Fli-1 is expressed primarily in hematopoietic and endothelial cells and can act either as an activator or repressor. It was first identified as the gene locus rearranged and overexpressed in 75% of Friend-murine leukemia virus-induced erythroleukemias (11). In humans, Fli-1 is fused to the EWS gene as a result of a chromosomal translocation t(11;22) in Ewing's sarcomas and neuroepithelioma (12). Complete disruption of the Fli-1 gene (Fli-1 −/−) in mice results in early embryonic death due to defects in hematopoietic and vascular development (13). Overexpression of Fli-1 in all tissues of transgenic mice results in death due to immunologic renal disease, and B cells from these mice have reduced levels of apoptosis and a prolonged cell survival (14).

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Correspondence: Kathleen M. Sakamoto, M.D., Ph.D., Division of Hematology-Oncology, Mattel Children's Hospital at UCLA, 10833 LeConte Ave., Los Angeles, CA, 90095; kms@ucla.edu.
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Consistent with these findings, overexpression of Fli-1 in primary erythroblasts results in enhanced myeloid cell survival through inhibition of differentiation and apoptosis (15).

We previously reported that Fli-1 binds the serum response element (SRE) of the *egr-1* promoter in myeloid cells stimulated with G-CSF (16,17). In gel shift experiments, Fli-1 recognizes a SRE that consists of an ETS binding site (EBS) and an adjacent SRF binding motif (CArG box), which is also sufficient to induce transcriptional activation in response to G-CSF. Fli-1 binding to the SRE is increased in G-CSF-treated cells. To determine whether the increase in Fli-1 binding results from increased Fli-1 expression, we examined the protein and RNA levels of endogenous Fli-1 in the murine myeloid leukemia cell line, NFS60. Furthermore, we examined Fli-1 protein levels in the presence of the protein synthesis inhibitor cycloheximide and performed pulse-chase experiments to determine the half-life of Fli-1 in the presence and absence of G-CSF.

In this article, we report that the steady state levels of Fli-1 protein are increased in response to G-CSF, independent of transcription, and that the half-life of Fli-1 protein is prolonged in G-CSF treated cells. Our results suggest that hematopoietic growth factor activation regulates the stability of transcriptional regulators, thereby promoting signals that determine myeloid cell fate.

METHODS

G-CSF time course analysis. NFS60 cells (kindly provided by Jim Ihle, St. Jude's Children's Hospital, Memphis, TN) were cultured in RPMI medium with FCS 10%, PCN/Strep, L-glutamine, and WEHI3B conditioned media containing IL-3 as previously described (16). For time course experiments, NSF60 cells were serum- and growth factor-starved in RPMI medium with BSA 0.5% for 15 h, followed by treatment with diluent (0.02% BSA in PBS) or G-CSF (10 ng/mL) for indicated times. Cells were harvested and nuclear and cytoplasmic or whole-cell extracts were prepared as previously described (16,18). For cycloheximide experiments, cells were starved for 15 h as described above and were treated with diluent or G-CSF in the presence or absence of cycloheximide (30 μg/mL) for 0, 30, 60, 120, and 180 min. Cells (10^6) were harvested at the indicated times and lysates were prepared by adding SDS-Laemmli buffer (250 mM Tris Base, 0.5% bromophenol blue, and 40% glycerol) containing 0.02% SDS and 0.048 mM β-mercaptoethanol), then boiling the sample for 5 min.

Western blot analysis. Nuclear and cytoplasmic extracts were prepared from NFS60 cells by the modified Dignam method (16,18). Twenty micrograms of protein from each sample were electrophoresed on a 10% SDS-polyacrylamide gel and trans-blotted onto nitrocellulose membrane. The blots were blocked in 2% milk in TBS-T and incubated with anti-SRF (0.5 mg/mL), anti-Fli-1 (0.5 mg/mL), or anti-actin (0.3 mg/mL) antisera (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with ECL Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ). To determine relative Fli-1 levels in samples, immuno-blots were stripped and incubated with anti-actin antisera. Densitometry analysis was used to compare the signals for both Fli-1 and actin for each sample, and to obtain relative value for Fli-1 levels.

Northern analysis. Cells (10^6) were serum- and growth factor-starved for 15 h, then stimulated with G-CSF for 0, 1, 2, 4, and 6 h. The cells were harvested, and total RNA was extracted by the Nonidet P-40 method (19). Twenty micrograms of total RNA from each sample was separated on a 1% formaldehyde gel, and was transferred to a nylon membrane. The membrane was hybridized with a 1.3 kb Fli-1 or actin cDNA labeled with [^{32}P]-dCTP as described (18).

Pulse-chase experiment. NFS60 cells (10^6 cells/mL) were incubated in RPMI 1640 medium without L-glutamine, L-cysteine, and L-methionine (ICN, Costa Mesa, CA) (supplemented with 2% fetal bovine serum, 87.6 μg/mL L-glutamine 200 mM, and 0.1 U/mL penicillin-streptomycin) for 12 h. The cells were pulse-labeled with ICN TRANS^{35}S-LABEL methionine and cysteine (stock concentration 10 mCi/mL) at 0.2 mCi/mL per 10^7 cells for 30 min. Cells were then washed and placed in regular RPMI culture medium with 10% FCS and treated with diluent (PBS, 0.2% BSA) or G-CSF (10 ng/mL) for the indicated time points. A total of 2×10^6 cells were harvested at 0, 2, 4, and 6 h after treatment with G-CSF or diluent control. The cell pellets collected were lysed at 4°C in lysis buffer containing 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5% NP40, 0.2 mM EGTA, 50 mM NaF, 1 mM PMSF, 1 μg/mL pepstatin A, and 10 μg/mL leupeptin. Lysates were incubated with 2 μg/μL anti-Fli-1 rabbit polyclonal antibody (Santa Cruz Biotechnology) for 1 h,

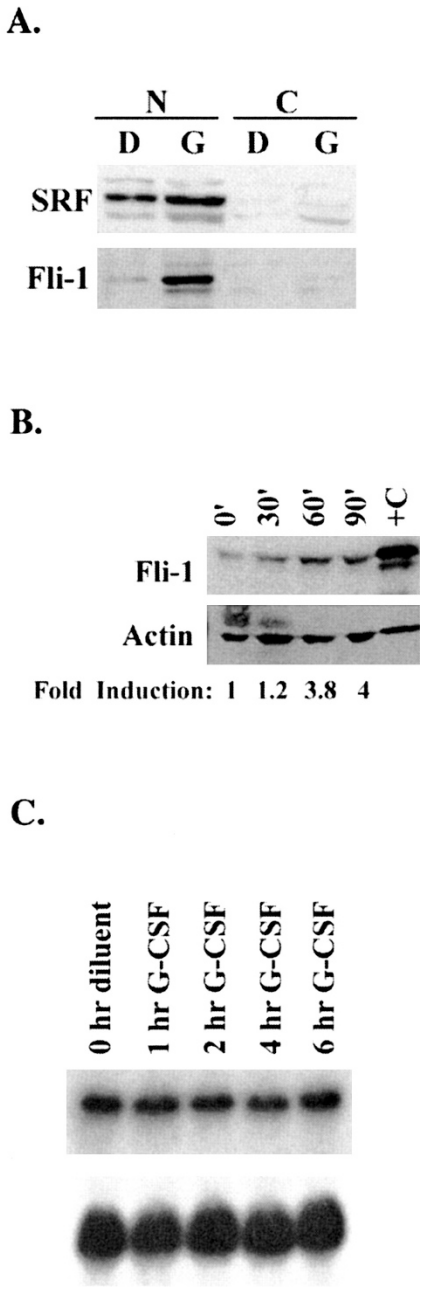

Figure 1. Fli-1 protein levels are increased in extracts from NFS60 cells treated with G-CSF. (*A*) Nuclear (*N*) and cytoplasmic (*C*) extracts were prepared by a modified Dignam method from quiescent NFS60 cells treated with diluent (*D*) or G-CSF (*G*). Western blot analysis was performed with anti-SRF or Fli-1 antisera. (*B*) Time course indicating Fli-1 protein levels increase in NFS60 cells treated with G-CSF. (*C*) Northern blot analysis demonstrating that Fli-1 RNA levels are constant.

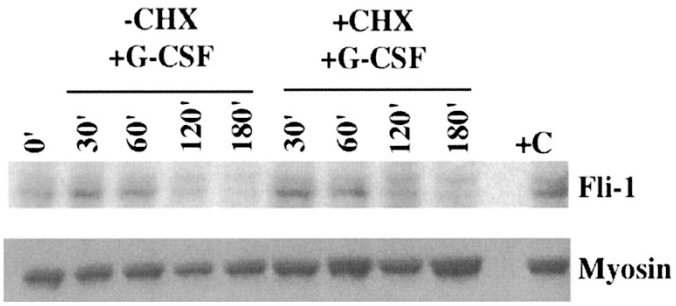

Figure 2. Increase in Fli-1 protein is dependent on protein synthesis. Fli-1 levels are increased in cells treated with cycloheximide (*CHX*) and G-CSF.

followed with protein A/G PLUS agarose beads (Santa Cruz Biotechnology) for 1 h. The beads were washed three times with wash buffer A containing 10 mM Tris-Cl pH 7.5, 0.1% Triton X, 0.15 M NaCl, 0.5 mM DTT, 1.5 mM $MgCl_2$, 1 mM PMSF, 10 μg/mL leupeptin, and 1 μg/mL pepstatin A; followed by three washes with wash buffer B containing 10 mM Tris-Cl pH 7.5, 0.5 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM PMSF, 10 μg/mL leupeptin, and 1 μg/mL pepstatin A. The protein was released from the beads by boiling in SDS-Laemmli buffer at 95°C for 5 min. An equal loading of the samples was subjected to 12% SDS-PAGE. The gel was dried with Bio-Rad Gel Dryer Model 583 (Bio-Rad, Hercules, CA) for 1 h, and exposed to phosphoscreen for >72 h. Band intensities were quantified using a phosphoimager (Typhoon 9000 series) and ImageQuant software (both from Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

RESULTS AND DISCUSSION

We previously demonstrated that endogenous SRF and Fli-1 proteins from NFS60 nuclear extracts bind the SRE-1 of the *egr-1* promoter in gel shift experiments (16). To examine the expression of Fli-1 and SRF in G-CSF– or diluent control–treated cells, we examined Fli-1 protein levels in nuclear or cytoplasmic extracts. Both Fli-1 and SRF proteins were expressed in nuclear extracts but not in cytoplasmic extracts (Fig. 1*A*). Whereas SRF protein levels were similar in nuclear extracts from G-CSF– (G) and diluent-treated (D) cells, Fli-1 protein levels were higher in nuclear extracts from G-CSF–treated (G) cells compared with diluent-treated (D) cells. We previously demonstrated that cytoplasmic extracts express JAK2 (data not shown). These results suggest that in nuclear extracts, Fli-1 protein levels increase after G-CSF stimulation of myeloid cells.

To characterize Fli-1 protein levels over time, NFS60 cells were serum- and growth factor–starved for 15 h and treated with G-CSF. We found a 3.8-fold increase in steady-state Fli-1 protein levels at 60 min and a 4.0-fold increase at 90 min after G-CSF stimulation (Fig. 1*B*). The increase in protein levels after G-CSF stimulation could be the result of either an increase in RNA and/or protein levels. To determine whether the increase in endogenous Fli-1 protein levels upon treatment with G-CSF occurs independent of transcription, we examined Fli-1 RNA levels by Northern blot analysis in NFS60 cells treated with G-CSF or diluent control (Fig. 1*C*). Our results demonstrated that Fli-1 transcript levels remained unchanged after G-CSF stimulation.

To test whether the increase in Fli-1 levels was dependent on protein synthesis, cells were treated with G-CSF in the presence and absence of the protein synthesis inhibitor, cycloheximide. At 120 min after G-CSF stimulation, we observed an increase in Fli-1 protein levels in cycloheximide-treated cells compared with untreated cells (Fig. 2). These results suggest that increased Fli-1 protein levels in response to G-CSF require protein synthesis.

To determine the half-life of Fli-1 in response to G-CSF, NFS60 cells were metabolically labeled with [^{35}S]-methionine

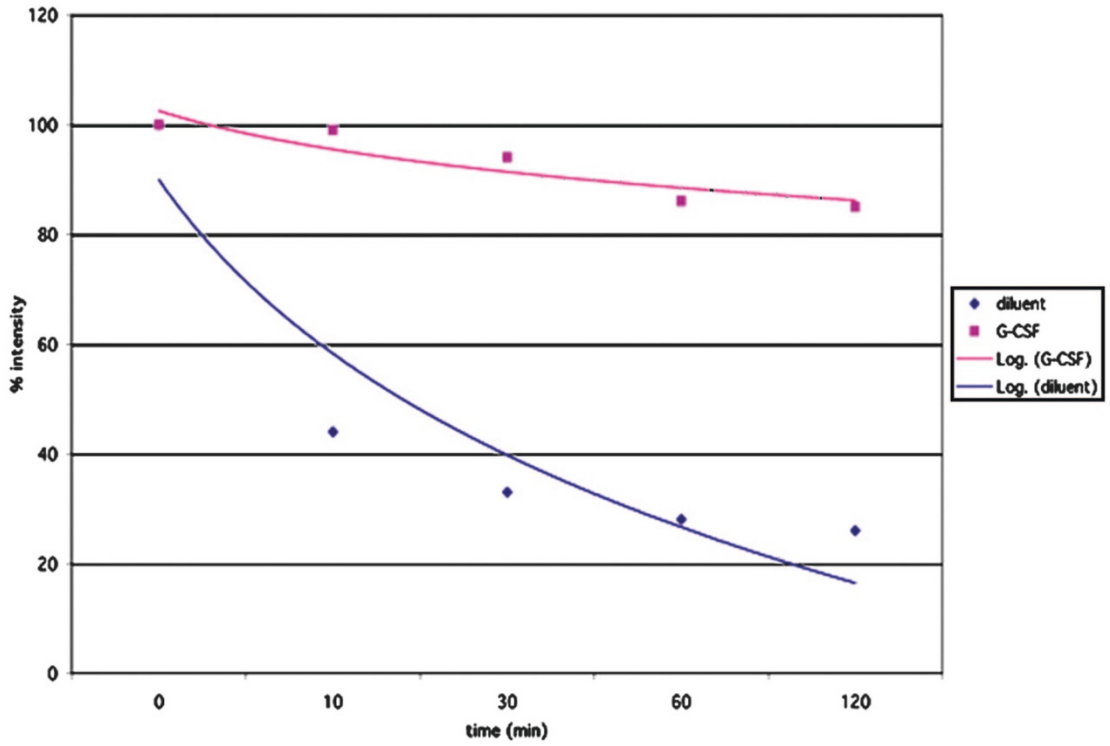

Figure 3. Half-life of Fli-1 is prolonged in G-CSF-treated NFS60 cells. Pulse chase analysis of Fli-1 in G-CSF– or diluent control–treated cells.

and cysteine. We observed the half-life of Fli-1 to be 15 min in diluent-treated cells and >120 min in G-CSF-treated cells (Fig. 3). Therefore, G-CSF treatment of myeloid cells results in stabilization of Fli-1 protein.

The regulation of Fli-1 protein is critical for normal myeloid cell development because aberrant expression of Fli-1 leads to malignant transformation in both humans and mice. In this report, we show that G-CSF stimulation of NFS60 cells results in prolonged half-life of Fli-1, which is most likely the result of posttranslational modification. Previous reports demonstrated that phosphorylation of myc, another important proto-oncogene, occurs through a Ras/Raf/MAPK-dependent pathway, resulting in stabilization the myc protein in response to serum stimulation (20). The short half-life (20 min) of the ETS protein family member, ETS2, has also been shown to increase dramatically (5- to 20-fold) in response to TPA (21). Thus, our results are consistent with previously published reports.

The modification of Fli-1 responsible for increased protein stability in G-CSF–treated cells is not known. We previously reported that SRE-1 transactivation occurs through a mitogen activated protein kinase (MEK1)/extracellular regulated kinase (ERK1 and 2)–dependent pathway, and phosphoinositol-1,3-kinase (PI3 K)–dependent pathway (17). Therefore, we hypothesize that the increase in Fli-1 stability in G-CSF-treated cells is a consequence of multiple kinase pathways converging on Fli-1, resulting in phosphorylation and stabilization of the protein. This would lead to subsequent regulation of appropriate target genes. Alternatively, acetylation, sumoylation, or localization of Fli-1 could influence stability in response to G-CSF. Further studies will be necessary to investigate the mechanism by which Fli-1 is modified during myeloid cell development.

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