Granulocyte Colony-stimulating Factor Induces egr-1 Up-regulation through Interaction of Serum Response Element-binding Proteins*

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Granulocyte colony-stimulating factor (G-CSF) stimulates proliferation and maturation of myeloid progenitor cells both in vitro and in vivo. We showed that G-CSF rapidly and transiently induces expression of egr-1 in the NFS60 myeloid cell line. Transient transfections of NFS60 cells with recombinant constructs containing various deletions of the human egr-1 promoter identified the serum response element (SRE) between nucleotides (nt) –418 and –391 as a critical G-CSF-responsive sequence. The SRE (SRE-1) contains a CArG box, the binding site for the serum response factor (SRF), which is flanked at either side by an ETS protein binding site. We demonstrated that a single copy of the wild-type SRE-1 in the minimal promoter plasmid, pTE2, is sufficient to induce transcriptional activation in response to G-CSF and that both the ETS protein binding site and the CArG box are required for maximal transcriptional activation of the pTE2-SRE-1 construct. In electromobility shift assays using NFS60 nuclear extracts, we identified SRF and the ETS protein Fli-1 as proteins that bind the SRE-1. We also demonstrated through electrophoretic mobility shift assays, using an SRE-1 probe containing a CArG mutation, that Fli-1 binds the SRE-1 independently of SRF. Our data suggest that SRE-binding proteins potentially play a role in G-CSF-induced egr-1 expression in myeloid cells.

Myeloid blood cell production is controlled by cytokines such as colony-stimulating factors (CSFs) and interleukins (ILs). Granulocyte colony-stimulating factor (G-CSF) stimulates survival, proliferation, and differentiation of granulocytic precursors and activation of neutrophils (1, 2). G-CSF mediates its cellular effects through binding the G-CSF receptor, a member of the cytokine receptor superfamily (2, 3).

Growth factor-mediated signals promoting cell proliferation or differentiation incite rapid induction of a family of genes termed immediate early genes (4, 5), which include c-fos (6) and c-jun (7), and the early growth response gene egr-1 (8) (also known as Tis 8, Krox 24, NFGIA, and zif268). Egr-1 is a ubiquitously expressed zinc finger transcription factor (59 kDa) (9) that can act to either positively or negatively regulate gene transcription (10, 11). Egr-1 has been demonstrated to be a critical upstream mediator of proliferation (12, 13), differentiation (14–17), and apoptosis (18, 19).

Treatment of myeloid cells with G-CSF results in rapid and transient expression of egr-1 independently of protein synthesis. G-CSF induces egr-1 expression and granulocyte differentiation in 32Del3 cells (20) and also stimulates proliferation and egr-1 expression in human UT-7 eop cells overexpressing the wild-type G-CSF receptor (21). The expression of egr-1, like that of other immediate early genes, is governed by preexisting regulatory proteins that are posttranslationally modified and thus activated upon receptor stimulation.

The precise signaling events that mediate expression of egr-1 in response to G-CSF in proliferative responses have not been elucidated. Identification of this pathway may provide insights into possible mechanisms that lead to the development of leukemia. It has been suggested that G-CSF selectively activates distinct early growth response genes through different Janus kinase-STAT proteins. For example, G-CSF stimulation of the early genes OSM, IRF-1, and egr-1 is independent on STAT5 activation, whereas activation of c-fos is STAT5-independent (21). Although STAT5 protein expression is induced in response to G-CSF, that STAT5 DNA recognition element has not been identified in the murine egr-1 promoter (21).

Signaling pathways that control egr-1 expression and myeloid cell proliferation have been examined for granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (22). The receptors for GM-CSF and IL-3, like G-CSF receptor, are also members of the cytokine receptor superfamily and regulate early myeloid development. GM-CSF- and IL-3-induced signals converge upon the cAMP response element in the egr-1 promoter in the factor-dependent human myeloid leukemia TF-1 cell line (22). egr-1 promoter sequences mediating G-CSF-induced egr-1 expression and myeloid proliferation, including the egr-1 promoter sites responsive to G-CSF, appear to be distinct from those involved in GM-CSF and IL-3 signaling, due to the different proteins activated by these proteins.

The goal of our study was to define G-CSF-responsive sequences of the egri-1 promoter and to identify interacting proteins in NFS60 cells. We show that egr-1 is rapidly and transiently expressed in NFS60 cells stimulated with G-CSF and that this activation occurs independently of protein synthesis. Transient transfections with recombinant egr-1 promoter constructs in NFS60 cells demonstrated that the CArG box and the
ETS protein binding site (EBS) are required for maximal transcriptional activation of egr-1 in response to G-CSF. Electromobility gel shift assays (EMSA) showed that SRF and Fli-1 bind the CArG and EBS between nucleotides (nt) –418 and –391 in the egr-1 promoter, respectively. Our experiments suggest that serum response element (SRE)-binding proteins may associate as a quaternary complex to maximally activate egr-1 transcription. Thus, signaling pathways activated by G-CSF may be distinct from those activated by GM-CSF or IL-3, suggesting a potential mechanism for specificity between growth factors that regulate myelopoiesis.

MATERIALS AND METHODS

NFS60 Cells—The murine myeloid leukemic factor-dependent NFS60 cell line was cultured in 1 x RPMI medium containing 10% fetal calf serum, penicillin (100 units/ml)-streptomycin (1 mg/ml) at a ratio of 1 unit/ml to 1 mg/ml, L-glutamine (2 mM), and gentamicin (10 mg/ml). Cells were maintained on IL-3 WEHI-3 conditioned media (1:100) in 10% BSA in phosphate-buffered saline. Cells were serum- and growth factor-starved for 18 h in RPMI-0.5% BSA. Twenty million cells were transfected with 20 μg of the specified egr-1 promoter/CAT construct or 20 μg of the specified minimal promoter pTE2/CAT construct and 5 μg of the pCMV-β-galactosidase plasmid (pCMV-βgal) (internal control). Transfected cells were resuspended in RPMI-0.5% BSA and stimulated with G-CSF (10 nM) or diluent control (0.02% BSA) for 3 h in RPMI-0.5% BSA. Half the lysates were assayed for CAT activity and the other half for βgal activity. The CAT assay was used to measure egr-1 promoter activity and was performed as described previously (22). The amount of acetylated and unacetylated [14C]chloramphenicol was determined by thin-layer chromatography and quantified by liquid scintillation counting. The βgal assay (Promega) was used as an internal control for transfection efficiency. Corrected fold stimulation was determined by dividing the percentage of acetylation of the G-CSF-stimulated cells by unstimulated (diluent) cells. Statistical analysis was performed using the JMP In program (SAS Institute Inc.).

EMSAs—The probes used for EMSA experiments included the egr-1 SRE-1 fragment (nt –600 to –7 of the putative transcription start site) which was gel-purified from the full-length human egr-1 genomic clone (24). Construction of plasmids containing the full-length or various deletions of the –600 egr-1 fragment (–480, –387, –235, –180, –116, and –56 nt) has been described (25).

FIG. 2. Human egr-1 promoter deletion constructs. The –600, –480, –387, –235, –180, –116, and –56 nt regions of the human egr-1 promoter were subcloned into the pCAT reporter plasmid. The CAT response element (CRE), SRE, SP-1, and EBS regulatory elements are depicted. egr-1 construct (20 μg) and 5 μg of CMV-β-galactosidase plasmid were transiently transfected by electroporation into serum- and factor-starved NFS60 cells. Cells were then treated with G-CSF (10 ng/ml) for 3 h, and the cell lysates were prepared and assayed for CAT or β-galactosidase activity. The fold induction, represented by the bars, was determined as described under "Materials and Methods." These data represent the average of three to seven experiments performed in duplicate or triplicate. A significant decrease in CAT stimulated reporter activity (*) was observed for the p-480 nt construct compared with the p-387 nt construct (p = 0.0024).
Fig. 3. EMSA of nuclear proteins that bind the SRE-1 oligonucleotide in the presence of SRF antibody. A, schematic representation of the -480 nt region of the egr-1 promoter. The SREs of the egr-1 promoter are depicted. The SRE-1 lies between nt -418 and -387 and is composed of a central CArG box and two EBSs. B, an SRE-1 oligonucleotide (0.1 µg) was labeled with [γ-32P]dATP and incubated with 20 µg of unstimulated (di) or 15 µg of G-CSF-stimulated (G) NFS60 nuclear extracts with a 200 x excess of nonspecific (NS) or specific (SP) unlabeled oligonucleotide. The diluent-treated extracts produced four gel shift bands/complexes (D, 1–4), and the G-CSF-treated extracts produced five gel shift bands/complexes (G, 1–5). C, the SRE-1 probe was incubated with 15 µg of G-CSF-stimulated nuclear extracts in combination with a 200 x excess of unlabeled oligonucleotide (nonspecific (NS), SRE-1, or CArG sequence) or with SRF antibody or IgG control. Gel shifts were electrophoresed on a 4% SDS-polyacrylamide gel. The bands represent reactions that were run on the same gel.

GAAAT[CCgAGATcG]G[GCAGGAGG]GATCCT-3' (di); reverse, 5'-CTAGAGGGGATC[CTTGCTGCTG][CCgAGATcG]G[ATTTCCCGG]GTCGCA-3', LmR (forward, 5'-AGCTTGCGAC[CCgAGATcG]G[GCAGGAGG]GATCCT-3'; reverse, 5'-CTAGAGGGGATC[CTTGCTGCTG][CCgAGATcG]G[ATTTCCCGG]GTCGCA-3'), and LmR'G (forward, 5'-AGCTTGCGAC[CCgAGATcG]G[GCAGGAGG]GATCCT-3'; reverse, 5'-CTAGAGGGGATC[CTTGCTGCTG][CCgAGATcG]G[ATTTCCCGG]GTCGCA-3'). Complimentary single-stranded oligonucleotides were synthesized, annealed, and end-labeled using [γ-32P]dATP and T4 polynucleotide kinase. Labeled probe was purified with Nuctrap Push Columns (Stratagene Cloning Systems, La Jolla, CA). Nuclear extracts were prepared by the modified Dignam method (26) from unstimulated (diluent) or G-CSF-stimulated NFS60 cells for 30 min. Protein concentrations were determined by the Bradford assay with Pierce protein assay reagents. Nuclear extracts (15–20 µg) were incubated with 0.1 µg of labeled probe in the presence of 1 µg of poly(dI-dC):(dI-dC) and 5 µg of BSA in 20 µl of gel shift buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 25% (v/v) glycerol) for 30 min on ice. Competitor oligonucleotides or antibodies were preincubated with the nuclear extracts for 30 min on ice prior to the addition of probe. Competitor oligonucleotides, including the nonspecific sequence, 69ALL, or wild-type or three mutant forms of the SRE-1 sequence, were added in 100- or 200-fold molar excess. Fli-1 antibody (2, 4, or 8 µg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used in supershift assays. Polyclonal rabbit IgG (Sigma) was used as the control antiserum (1.1 µg).
ng/µl. After incubation periods, the samples were loaded onto a 4% polyacrylamide gel and run at 100 V in 0.4× TBE. Gels were dried and exposed to film at −70 °C.

RESULTS

NFS60 Cells Are Dependent on G-CSF for Proliferation—We demonstrate that NFS60 cells are a growth factor-dependent myeloid cell line (data not shown). Furthermore, NFS60 cells proliferate in response to G-CSF in a dose-dependent manner (27, 28). Therefore, NFS60 cells provide a good model in which to examine G-CSF-induced proliferative signals.

G-CSF Induces Rapid and Transient Expression of egr-1 in NFS60 Cells—Rapid and transient expression of egr-1 has been previously shown to occur in response to GM-CSF in myeloid leukemic TF-1 cells (22). To demonstrate egr-1 expression in NFS60 cells in response to G-CSF, Northern blot analysis was performed. Northern blot analysis with RNA from NFS60 cells stimulated with G-CSF for 0, 30, 60, 90, and 120 min demonstrated a rapid and transient induction of egr-1 (Fig. 1). Expression of egr-1 was not induced in diluent-treated cells (0 min). Accumulation of egr-1 RNA was observed within 30 min and was no longer observed at 60 min following G-CSF treatment. Stimulation of cells for 60 min with G-CSF or 12-O-tetradecanoylphorbol-13-acetate in the presence of the protein synthesis inhibitor cycloheximide resulted in induction of egr-1. 12-O-tetradecanoylphorbol-13-acetate has been previously shown to induce egr-1 expression within 60 min of cell treatment (8). These results were confirmed in two independent experiments. Our results demonstrate that egr-1 expression occurs rapidly and transiently in cells stimulated with G-CSF and that induction of egr-1 occurs independently of protein synthesis.

The −600 and −480 nt Region of the egr-1 Promoter Contains G-CSF-responsive Sequences—To identify the egr-1 promoter sequences that are responsive to G-CSF signaling, NFS60 cells were transiently transfected with egr-1 promoter constructs. Serum- and growth factor-starved NFS60 cells were transfected with constructs containing −600, −480, −387, −235, −180, −116, or −56 nt of the human egr-1 promoter (Fig. 2) and pCMV-βgal for measurement of transfection efficiency.

We previously showed that the −56 nt region of egr-1 contains minimal activity that is equal to the pCAG empty vector (22). We have therefore used p-56 CAT as the vector control in our experiments. In response to G-CSF, the −600 and −480 nt egr-1 constructs demonstrated maximal stimulation (Fig. 2) at 9.0-fold activity relative to p-56 CAT control vector. Deletion of nucleotides between −480 and −387 resulted in a 6-fold decrease in transcriptional activation (p = 0.0024; Fig. 2), and further deletion to nt −116 did not reduce this activity. In diluent-treated cells, the constructs had basal activities that were not statistically significantly different from that of the control vector (data not shown). All transfections were performed in duplicate or triplicate, representing an average of three to seven experiments. These results indicate that the region between −480 and −387 nt of the egr-1 promoter contains critical sequences required for maximal transcriptional activation of egr-1. The region between nt −480 and −387 contains a single SRE, with a central CArG box flanked by EBSs, which we have called SRE-1 (Fig. 3A). Transfections with a construct containing −418 nt of the egr-1 promoter (Fig. 3A) showed similar activity levels with the p-480 nt construct (data not shown). These results suggested that the SRE, between nt −418 and −391 (SRE-1), may contain a critical transcription factor binding site regulating G-CSF-induced transcription of egr-1.

Nuclear Extracts from NFS60 Cells Contain Proteins That Recognize the SRE-1 Sequence—Previous studies have identified SRF binding to the CArG box of SREs in the promoters of immediate early genes (29–32). To determine whether endogenous SRE-binding proteins in nuclear extracts from NFS60 cells interact with the wild-type SRE-1 sequence between nt −418 and −391 of the egr-1 promoter (Fig. 3A), EMSAs were performed. Nuclear extracts prepared from diluent- or G-CSF-treated NFS60 cells were incubated with the SRE-1 oligonucleotide probe with an excess of unlabeled specific or nonspecific competitor sequence. Probe and extracts were also incubated with 2–8 µg of Fli-1 antibody. SRF antibody and IgG were used as positive and negative controls, respectively. Gel shift bands 1 and 2 represent two distinct SRF-containing complexes. IA and IB indicate bands formed upon addition of Fli-1 antibody.

Fig. 4. EMSA identification of EBS proteins that bind SRE-1.

An oligonucleotide SRE-1 sequence (0.1 µg) was labeled with [γ-32P]dATP and used as probe in EMSA experiments. The probe was incubated with 15 µg of G-CSF-stimulated nuclear extract and a 200 × excess of nonspecific (NS) unlabeled competitor sequence. Probe and extracts were also incubated with 2–8 µg of Fli-1 antibody. SRF antibody and IgG were used as positive and negative controls, respectively. Gel shift bands 1 and 2 represent two distinct SRF-containing complexes. IA and IB indicate bands formed upon addition of Fli-1 antibody.
taining the CArG sequence (Santa Cruz Biotechnology Inc.) resulted in a supershifted band that co-migrates with the supershifted band seen with the SRE-1 probe (Fig. 3C, lane 11). Furthermore, an excess of unlabeled CArG oligonucleotide alone specifically competed bands 1 and 2 (Fig. 3C, lane 3), suggesting that these bands are SRF-containing complexes. Addition of the SRF antibody in EMSAs using diluent-treated extracts also resulted in a supershifted band pattern (data not

**Fig. 5.** Stimulation of pTE2 constructs containing the SRE-1 sequence. 

**A.** pTE2 oligonucleotide constructs containing one copy of the wild-type or mutant SRE-1 sequences (5' EBS mutant, L'R; 3' EBS mutant, LR; 5' and 3' EBS mutant, L'R'; and CArGm/BglII) were made. 

**B.** The pTE2 constructs (20 μg) were individually transfected into NFS60 cells with CMV-bgal (5 μg) in NFS60 cells serum- and factor-starved for 18 h. Cells were then stimulated with diluent (phosphate-buffered saline with 0.02% BSA) or G-CSF (10 ng/ml). The fold induction by G-CSF was determined as described under "Materials and Methods" and represents an average of 3–11 experiments performed in duplicate or triplicate. A significant increase in pTE2-SRE-1 activity (*) was observed as compared with the pTE2 empty vector (p < 0.001), and the pTE2 mutant constructs (L'R, p = 0.0381; LR, p = 0.0022; L'R', p = 0.0056; and CArG, p = 0.0132). 

**C.** Gel shift competition assays were performed to determine CArG mutations that inhibit SRF binding. An oligonucleotide SRE-1 sequence (0.1 μg) was labeled with [γ-32P]dATP and used as probe in EMSA experiments. The probe was incubated with 15 μg of G-CSF-stimulated nuclear extracts and a 200× excess of nonspecific (NS) oligonucleotide or an SRE-1 sequence with one of three different CArG mutations (GG deletion, GG to TT substitution, or BglII substitution). The arrow indicates the gel shift band (band 2) that is not competed by an excess of unlabeled CArG mutant oligonucleotide.
shown). These results were confirmed in two separate experiments. SRF binding to the SRE has been shown to be enhanced upon SRF phosphorylation (33, 34), yet it has also been shown to bind the SRE constitutively (35–38). Our data indicate that SRF binds the SRE-1, and the supershift band corresponds with the band observed with SRF binding to the CArG probe. Thus, these results indicate that SRF binds the CArG sequence of SRE-1 in G-CSF-treated nuclear extracts.

**Fli-1 in NFS60 Nuclear Extracts Binds the EBS in SRE-1 of the egr-1 Promoter**—A previous report has demonstrated that Fli-1 is one of the ETS proteins that recognizes the EBS of SRE-1 in the egr-1 promoter (39). To identify ETS proteins in NFS60 nuclear extracts that bind the EBS of SRE-1, EMSA experiments were performed using antibodies to various ETS proteins. Addition of Fli-1 antibody to G-CSF-stimulated extracts resulted in the disappearance of band 1 and the formation of two supershifted bands, 1A and 1B (Fig. 4, lanes 1–3). This suggests that band 1A represents a complex composed of both SRF and Fli-1 bound to the SRE-1 probe. Band 1B may represent a complex of SRF and Fli-1 with additional proteins. Addition of Fli-1 antibody in EMSAs using diluent-treated extracts resulted in a very weak supershift band pattern under certain conditions (data not shown). Addition of antibodies to other ETS proteins, including Elk-1, Sap1a/b, PU.1, EII-1, ETS-1/2, ERG-1/2, and PEA3, did not produce a change in the gel shift pattern in either diluent- or G-CSF-treated extracts (data not shown). Our data suggest that in addition to SRF, Fli-1 also binds the SRE-1 in the egr-1 promoter in G-CSF-stimulated extracts.

**The SRE-1 Sequence Is Sufficient to Induce Transcription in Response to G-CSF**—Ternary complexes composed of SRF and ETS protein family members have been shown to regulate the expression of many immediate early gene SREs (32, 40–47) and may also regulate G-CSF-induced egr-1 expression. To determine whether the SRE-1 sequence is sufficient to induce egr-1 transcriptional activation in response to G-CSF, NFS60 cells were transiently transfected with a construct containing a synthetic oligonucleotide representing a single human SRE-1 in the pTE2 vector that contains a heterologous TK promoter and the CAT gene. pTE2 constructs with mutations within the SRE-1 were also prepared (Fig. 5A). The mutant SRE-1 constructs included a 3-base substitution within the CArG core consensus binding sequence (CArGm/BgII) and a GG to TT substitution within the ETS consensus binding site (GGA) at the left (LmR), the right (LRm), or both the left and right (LmRm) EBSs of SRE-1.

Upon G-CSF treatment, the pTE2 SRE-1 wild-type construct demonstrated a 3.5-fold induction compared with the empty vector (p < 0.001; Fig. 5B). Upon G-CSF stimulation, the pTE2-CArGm/BgII, LmR, LRm, and LmRm constructs behaved similarly to vector control, but all demonstrated reduced induction levels as compared with pTE2 SRE-1 (p = 0.0132, 0.0381, 0.0022, and 0.0056, respectively; Fig. 5B). In diluent-treated cells, the basal activity of the pTE2-CArGm/BgII, LmR, LRm, and LmRm constructs demonstrated percentage of acetylation values similar to the pTE2 empty vector, ranging from 1.5 to 0.82%, respectively (data not shown). These experiments were repeated 3–11 times and were performed in triplicate. The CArG box mutation (pTE2-CArGm/BgII) also efficiently inhibited competition of the SRF/SRE gel shift band (gel shift band 2) in EMSA experiments (Fig. 5C, lanes 2 and 3). Mutations within the EBS core consensus sequence at the 5′ (LmR), 3′ (LRm), or both 5′ and 3′ EBS (LmRm) were previously shown to inhibit ETS protein binding (39). Our data suggest not only that the SRE-1 is sufficient to induce transcriptional activation in response to G-CSF but also that the CArG box and both the 5′ and 3′ EBSs are required for maximal stimulation of the pTE2 SRE-1 construct. Ternary complexes composed of SRF and ETS protein family members have been shown to regulate the expression of many early gene SREs (32, 40–47), and they appear to regulate G-CSF-induced egr-1 expression.

**Fli-1 Binds SRE-1 Independently of SRF**—In vitro translated Fli-1 protein has previously been shown to bind the SRE-1 sequence in the murine egr-1 promoter independently of SRF (39). To determine whether Fli-1 in nuclear extracts from NFS60 cells requires SRF for binding to SRE-1, gel shifts were performed with nuclear extracts and a wild-type SRE-1 or a SRE-1 probe containing a mutation in the CArG box (CArGm/BgII; Fig. 6). In the presence of nonspecific competitor, bands 3–5 were observed with nuclear extracts from G-CSF-stimulated cells (Fig. 6, lane 7). The absence of bands 1 and 2 indicates that SRF cannot bind the CArG mutant probe. Use of the unlabeled mutant probe sequence and the wild-type SRE sequence in excess resulted in competition of all gel shift bands, due to the presence of intact EBSs (Fig. 6, lanes 8 and 9). The addition of SRF antibody and IgG control did not result in a supershifted band, whereas the addition of Fli-1 antibody resulted in a band of slower mobility (Fig. 6, lanes 10–12). Our data indicate that Fli-1 in nuclear extracts from myeloid cells can bind SRE-1 independently of SRF. Furthermore, although Fli-1 can bind SRE-1 independently of SRF, our transfection data suggest that both SRF- and ETS-binding protein(s) are required for maximal transcriptional activation of egr-1 in response to G-CSF.

**Fli-1 Binds to the 5′ EBS of SRE-1**—ETS proteins have been demonstrated to be sequence-specific transcription factors. In vitro translated Fli-1 was shown to specifically bind the murine egr-1 at the 5′ EBS of SRE-1 (39). To determine whether one or both EBSs are bound by endogenous Fli-1 in nuclear extracts from NFS60 cells, gel shifts using SRE-1 probes containing an intact CArG box and a mutation either in the 5′ (LmR) or the 3′ (LRm) EBS were performed, using G-CSF-stimulated nuclear extracts (Fig. 7A). The LmR probe, in the presence of nonspecific competitor, failed to form band 1 (Fig. 7A, lane 5). The absence of band 1, which represents the Fli-1/SRF-probe complex.
G-CSF and SRE-binding Proteins

FIG. 7. EMSA of nuclear proteins that bind an EBS mutant SRE-1 oligonucleotide. A, an SRE-1 labeled probe (0.1 μg) containing a wild-type CARG box or a mutation either in the SRE-1, LmR, or LRm EBS site was incubated with 15 μg of G-CSF-stimulated nuclear extracts in combination with a 200 μM excess of competitor sequences (SRE-1, LmR, or LRm) or SRF antibody (Ab), Fli-1 antibody, or IgG control. Gel shift band 1 represents an SRF-containing complex, and band 2 represents an SRF/Fli-1-containing complex. B, a left EBS probe containing the SRE-1, LmR, or LRm EBS site was incubated with 15 μg of G-CSF-stimulated nuclear extracts in combination with a 200 μM excess of competitor sequences (SRE-1, LmR, or LRm) or SRF antibody (Ab), Fli-1 antibody, or IgG control. C, a right EBS probe containing the SRE-1, LmR, or LRm EBS site was incubated with 15 μg of G-CSF-stimulated nuclear extracts in combination with a 200 μM excess of competitor sequences (SRE-1, LmR, or LRm) or SRF antibody (Ab), Fli-1 antibody, or IgG control. Gel shift bands 1, 3, and 4 were supershifted with the addition of SRF antibody, whereas bands 2, 5, and 6 were supershifted with the addition of Fli-1 antibody. The presence of band 1 confirms that Fli-1 binds the 5′ EBS. The LRm mutant probe in the presence of nonspecific competitor formed gel shift bands 1–5 (Fig. 7A, lane 10). The presence of band 1 confirms that Fli-1 binds the 5′ EBS. Addition of an excess of unlabeled LRm mutant probe sequence competed all the bands. Addition of SRF antibody resulted in the expected supershift pattern (Fig. 7A, lane 13). The SRE-1 probe, upon the addition of SRF and Fli-1 antibody, resulted in supershifted bands (Fig. 7A, lanes 2–4). These results were observed in two independent experiments. These results suggest that the 3′ EBS may inhibit the Fli-1 antibody interaction with Fli-1.

To test our hypothesis that Fli-1 binds the 5′ EBS of SRE-1, we performed EMSA experiments with the 5′ EBS alone as the probe. Addition of Fli-1 antibody to G-CSF-treated nuclear extracts and the 5′ EBS probe resulted in a supershifted band (Fig. 7B, lanes 9–11), but the addition of Fli-1 antibody to diluent-treated extracts did not (Fig. 7B, lanes 3–5). We performed similar gel shift experiments using the 3′ EBS as probe. In three independent experiments, addition of Fli-1 antibody to G-CSF-treated nuclear extracts and the 3′ EBS probe resulted in either a weakly supershifted band or no supershifted band (Fig. 7C, lanes 4–6). Thus, our data suggest that in G-CSF-stimulated extracts, Fli-1 predominantly binds the 5′ EBS of SRE-1 and that SRF can bind independently of either EBS.

DISCUSSION

Growth factor induction of egr-1 expression is important for proliferative and differentiation responses in normal and leukemic myeloid cells. Studies indicating a role for Egr-1 in growth response have been reported (25, 48–50). To elucidate the molecular events regulating myeloid cell proliferation, we examined the mechanism by which G-CSF-induced signals lead to an increase in egr-1 transcription. We identified an SRE complex bound by SRF and Fli-1, which mediates egr-1 induction in response to G-CSF. ETS proteins, in association with other DNA-binding proteins, have been previously suggested to play a role in hematopoiesis, including the regulation of genes involved in myeloid (22) and lymphoid cell development (51).

Mitogens and differentiating factors induce expression of various immediate early genes (egr-1, c-fos, c-jun, pip92, etc.) through the activation of SREs (32, 40–46). The trans-activation of many early genes containing SREs is regulated by complexes composed of SRF and ETS protein family members (32, 40–47). A ternary complex of SRF and Elk-1 or Sap1 bound to SRE has been shown to induce expression of both the c-fos (40–42, 46) and egr-1 genes (47). ETS proteins generate selective transcriptional responses through their specific protein partnerships and their sequence-specific DNA binding (52). It has been shown that the in vitro-translated SRF and ETS proteins Elk-1, Sap1, Fli-1 and EWS-Fli-1 bind SRE-1 of the murine egr-1 promoter (39). Fli-1 and EWS-Fli-1 were demonstrated to exclusively bind the 5′ EBS box of SRE-1, whereas Sap1 and Elk-1 bind both the 5′ and 3′ EBS (39). We have shown that endogenous SRF and Fli-1 proteins also bind extracts in combination with a 200 μM excess of competitor sequences (nonspecific (NS) or 3′ EBS (SP)) or the indicated amounts of Fli-1 antibody or IgG control. The left EBS probe was incubated with 15 μg of G-CSF-stimulated nuclear extracts and 4 μg of Fli-1 antibody (+ control).
the SRE-1 of the human *egr-1* promoter, with Fli-1 predominately binding the 5′ EBS (Fig. 7). The inability of Fli-1 antibody to produce a supershift with the LR monument in our experiments may be due to a change in the DNA/protein conformation, which may inhibit the Fli-1 epitope from being exposed to the Fli-1 antibody. Furthermore, the 3′ EBS as probe in EMSA experiments produced less intense gel shift bands as compared with the 5′ EBS as probe (Fig. 7C). This may reflect less protein binding to the 3′ EBS overall. Therefore, although Fli-1 may bind the 3′ EBS, it does not appear to bind strongly, supporting our hypothesis that 5′ EBS is the predominant Fli-1 binding site. We also did not identify SRE-1 binding by Elk-1 or Sap1 in EMSAs with nuclear extracts from NF560 cells (data not shown).

The binding of ETS proteins to the SRE has been shown to occur in both an SRF-dependent and an SRF-independent manner. We demonstrated that SRF can bind an SRE-1 oligonucleotide probe in both unstimulated (data not shown) and G-CSF-stimulated nuclear extracts (Fig. 3B) and that it can bind in the absence of either the 5′ or 3′ EBS (Fig. 7A). SRF binding to the SRE of the c-fos promoter is required for the recruitment of Elk-1 and Sap1 to the EBS sequences (53). However, binding of Elk-1 and Sap1 to the *egr-1* promoter does not require prior assembly of an SRF/SRE binary complex (39). We have also demonstrated that endogenous Fli-1 protein does not require SRF for binding to the 5′ EBS of *egr-1* SRE, which is likely due to this sequence being a high affinity binding site for Fli-1.

Induction of *egr-1* gene expression has been shown to be regulated by SREs during B-cell activation (54), myeloid cell proliferation (22), and monocyte (55) and preadipocyte (47) differentiation. In most studies, inducibility of *egr-1* promoter activity has been localized to six SREs within −420 nt of the *egr-1* promoter, with SRE-1 being the 5′-most element. Although most studies suggest that the individual SREs contribute equally to the induction of *egr-1* expression (25, 31, 56, 57), several studies have found regulatory roles for specific *egr-1* SREs (54, 55, 58, 59). Our data indicate that the 5′-most SRE (SRE-1), containing the central CARG box and flanking ETS boxes, is sufficient and necessary when isolated from the remainder of the *egr-1* promoter sequences. However, experiments with constructs containing site-directed mutations of SRE-1 in the context of the −420 nt *egr-1* promoter region indicated that SRE-1 was not required for maximal *egr-1* expression (data not shown). Our results suggest that additional proteins recognizing SRE motifs 3′ of SRE-1 between nt −369 and −326 or other elements may also associate with SRE-binding proteins in response to upstream signals.

Transcriptional activation of other immediate early genes occurs through signaling cascades targeting SRF or its associated ETS proteins. SRF is phosphorylated in response to serum and growth factors, stress stimuli, and tumor-promoting agents (34, 60, 61). SRF phosphorylation has been demonstrated to increase SRF DNA binding affinity and to increase ternary complex formation with ETS proteins (33, 34). However, several groups have reported that SRF constitutively binds the SRE, with no change in SRE binding patterns upon growth factor or serum stimulation (35–38). The role of ternary complex factors Elk-1 and Sap1 as the direct targets of signaling cascades responsible for regulating early gene expression has been more extensively studied. Transcriptional activation of the c-fos promoter by growth factors and stress stimuli has been shown to be mediated through phosphorylation of Elk-1 and Sap1 by the mitogen-activated protein kinase-independent kinases also phosphorylate Elk-1, thereby inducing SRF ternary complex trans-activation of the pip92 immediate early gene (32, 44).

The results presented here suggest that SRF and Fli-1 and/or an unidentified ETS protein form a ternary or quaternary complex on SRE-1 of the *egr-1* promoter, which is required for regulation of *egr-1* transcription. We show that the *egr-1* SRE-1 complex is bound by SRF, regardless of growth factor stimulation. This is consistent with constitutive binding of SRF to the c-fos promoter (35). Furthermore, we demonstrated that in supershift experiments, Fli-1 binds the SRE-1 very weakly in diluent-treated extracts and more strongly in G-CSF-stimulated extracts. This may reflect a posttranslational modification, such as phosphorylation, which increases the affinity of Fli-1 binding for SRE or which promotes Fli-1/SRF complexes to associate with other factors of the TFIID complex. We speculate that G-CSF induces changes in the electrophoretic mobility of SRF-containing complexes through the binding of a phosphorylated form of Fli-1 and/or SRF proteins. It has recently been demonstrated that calcium-dependent phosphorylation of Ets-1 inhibits Ets-1 DNA binding activity (70). However, phosphorylation of Fli-1 through the Ras/mitogen-activated protein kinase signaling pathway may potentially stimulate Fli-1 binding to SRE-1. The role of Fli-1 in G-CSF signal transduction is currently under investigation. In preliminary co-transfection experiments with a Fli-1 expression construct and pTE2-SRE-1, Fli-1 does not increase *egr-1* transcription in NF560 cells (data not shown). This may be due to rate-limiting kinases or to the fact that the pTE2-SRE-1 construct is maximally active at 3-fold stimulation.

We previously examined the signaling pathways that regulate myeloid cell proliferation in response to GM-CSF and IL-3, using the *egr-1* gene as an end point (22). The receptors for GM-CSF and IL-3, like those for G-CSF, are members of the cytokine receptor superfamily and transduce signals through a common β subunit. We reported previously (22) that phosphorylation of the cAMP response element-binding protein is critical for the induction of *egr-1* in response to GM-CSF. In this study, we show that G-CSF activates *egr-1* transcription through an SRE. Thus, our findings indicate that different signaling pathways converge on distinct promoter regions of *egr-1*, demonstrating a possible mechanism that determines specificity of myeloid growth factors and their actions on proliferation.

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