Involvement of Immediate-early Gene Expression in the Synergistic Effects of Steel Factor in Combination with Granulocyte-Macrophage Colony-stimulating Factor or Interleukin-3 on Proliferation of a Human Factor-dependent Cell Line*

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Steel factor (SLF) synergizes with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) to stimulate proliferation of human factor-dependent cell line, MO7e. To elucidate molecular mechanisms underlying this synergism, induction of immediate-early genes was studied. Treatment of MO7e cells with SLF, GM-CSF, and IL-3 induced/enhanced expression of c-fos, junB, egr-1, and c-myc genes. SLF treatment of MO7e cells led to higher expression of c-fos, junB, and egr-1 genes than did treatment with GM-CSF or IL-3. However, GM-CSF and IL-3 had more prolonged effects on enhancement of the c-myc gene than SLF. Using optimal dosages for cell proliferation, induction of c-fos and junB was greater than additive with SLF plus GM-CSF or IL-3, as compared with each factor alone. Using suboptimal amounts of SLF with optimal GM-CSF or IL-3, induction of c-fos, junB, egr-1, and c-myc genes was greater than additive. De novo protein synthesis was not required for greater induction of these immediate-early genes by the combination of SLF plus GM-CSF. Based on nuclear run-on and actinomycin D experiments, the data suggest that the synergistic effects of SLF plus GM-CSF on the induction of immediate-early genes may be mediated in part at the level of transcription and mRNA stabilization for c-fos, at the level of mRNA stabilization for junB, and at the level of transcription for egr-1.

Induction of cell proliferation triggers expression of a set of genes termed immediate-early genes (reviewed in Ref. 1). Among these, genes encoding transcription factors, such as fos (2–5) and jun family (6–8), egr-1 (9), and c-myc (10) genes, are of special interest. The products of fos and jun genes form a heterodimeric transcription factor complex, called AP1, and activate genes that contain 12-O-tetradecanoylphorbol-13-acetate-responsive elements. Involvement of Fos and Jun family proteins in cell cycle progression has been demonstrated by using anti-sense RNA (11) or by microinjection into cells of antibodies raised against Fos and Jun family proteins (12).

egr-1 (also known as zif268, NGFI-A, TIS8, and krox24) encodes a zinc-finger-containing protein, which binds specifically to DNA containing the sequence GCGGGCGC (13) and activates transcription (14). The egr-1 gene is induced in a variety of cell types by growth factors and depolarizing agents (15–17). Recent findings that the wt1 gene (18), a putative tumor suppressor gene located at the Wilms' tumor locus, encodes a zinc-finger-containing protein that binds to the egr-1 consensus sequence (19) and represses transcription (20) further suggest a role for the egr-1 gene product in cell proliferation.

Steel factor (SLF), also known as mast cell growth factor, stem cell factor, or c-kit ligand, is a glycosylated polypeptide that exists in soluble and membrane-bound forms (21–24). cDNA that encodes SLF has been isolated (23, 24), and the human SLF gene has been mapped to chromosome 12q22-24 (25). SLF has been implicated in a number of important developmental roles (reviewed in Ref. 26), and is a potent costimulating cytokine that acts synergistically with hematopoietic colony-stimulating factors (CSF) to stimulate colony formation of hematopoietic progenitor cells (21–23, 27). SLF codes for the ligand of the c-kit proto-oncogene product that has intrinsic tyrosine kinase activity (28). Binding of SLF to the c-kit product is followed within minutes by a signal transduction cascade involving protein phosphorylation (29–31). However, the mechanisms involved in the synergistic effects of SLF with other cytokines in the stimulation of cell proliferation does not, so far, appear to be explained by modulation of CSF receptors (32) or by subsequent phosphorylation of proteins (31). Thus, we chose to evaluate nuclear events induced by SLF and to elucidate possible molecular mechanisms underlying synergism between SLF plus either granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) by focusing on immediate-early gene expression.

EXPERIMENTAL PROCEDURES

Cytokines—Purified recombinant human SLF, recombinant human GM-CSF, and recombinant human IL-3 were kind gifts from Dr. Douglas E. Williams (Immunex Corporation, Seattle, WA) (22, 24, 27).

Cells—The human factor-dependent cell line, MO7e, is a subline of MO7 cells, originally isolated from a patient with acute megakaryocytic leukemia, and was obtained from Aggie Ciarletta (Genetics

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† The abbreviations used are: SLF, steel factor; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-3, interleukin-3; kb, kilobase(s).
Northern Blot Analysis—After 18 h of factor starvation in RPMI 1640 medium containing 0.5% bovine serum albumin, cytoplasmic or total cellular RNAs (34) were prepared from cells with or without SLF, GM-CSF, and IL-3 stimulation. Fifty ng/ml of SLF, 500 units/ml of GM-CSF, and 1000 units/ml of IL-3 were used, except for dose response experiments. These concentrations have been shown to be the maximally effective concentrations for inducing proliferation of MO7e cells when each cytokine is used alone (32). Northern hybridization was performed using either 10 or 20 µg of RNA (34).

DNA Probes—Human c-fos (2) and c-myc (pG1-5'-c-myc) probes were obtained from the American Type Culture Collection. Human glyceraldehyde-3-phosphate dehydrogenase and c-jun oligonucleotide probes were obtained from Oncogene Science Inc. (New York). Human junB (35), human γ-actin (36), and mouse egr-1 (37) probes were kind gifts from Dr. Jochen Schütte (Innere Universitätsklinik und Poliklinik, Essen, Germany), Dr. Winston A. Saker (UCLA School of Medicine, Los Angeles, CA), and Dr. Harvey R. Herschman (UCLA School of Medicine, Los Angeles, CA). PstI fragment (1 kb) of c-fos, PstI-Styl fragment (1.2 kb) of junB, EcoRI fragment (1.4 kb) of egr-1, SstI fragment (1.6 kb) of c-myc, and HindIII-PstI fragment (0.4 kb) of γ-actin gene were used as probes for Northern hybridization and nuclear run-on transcription assays. c-fos, junB, egr-1, and c-myc probes were radiolabeled by the method of random priming (38) using [α-32P]CTP. Glyceraldehyde-3-phosphate dehydrogenase and c-jun oligonucleotides were radiolabeled by using T4 polynucleotide kinase and [γ-32P]ATP (34).

Nuclear Run-on Transcription Assay—Assay of transcription rate in nuclei was performed as described using 5 × 10⁵ cells/group (39). Two micrograms of specific inserts for c-fos, junB, egr-1, and γ-actin genes were used as probes.

RESULTS

We addressed the potential mechanism of synergism by examining expression of several immediate-early genes in GM-CSF-, IL-3-, and SLF-treated MO7e cells (Figs. 1 and 2). As previously reported (32), SLF, GM-CSF, or IL-3 stimulated proliferation of MO7e cells, and SLF synergized with either GM-CSF or IL-3 in this effect (data not shown). In that study (32), synergy was defined as an effect in the presence of two cytokines that is greater than the combined effects of the individual cytokines. No expression of c-jun was detected regardless of the treatment used (data not shown). In three separate experiments, the levels of c-myc, c-fos, junB, and egr-1 gene expression were enhanced by SLF, GM-CSF, or IL-3 treatment. (The results of one experiment is shown in Fig. 1; the relative expression of another experiment is shown in Fig. 2.)

Levels of c-myc mRNA were maximal at 1 h after factor treatment. Either GM-CSF or IL-3 led to slightly greater and more prolonged enhancement of c-myc gene expression than SLF. In three experiments, combined treatment of cells with SLF plus either GM-CSF or IL-3 led to greater expression of c-myc than that seen with each factor alone.

c-fos mRNA rapidly increased following factor treatments and reached maximal levels at 30 min. Levels of c-fos returned to base line by 2 h. SLF induced higher levels of c-fos expression than either GM-CSF or IL-3. At 30 min after induction, combined treatment of cells with SLF plus either GM-CSF or IL-3 led to a 9–25-fold higher expression of c-fos mRNA than with either GM-CSF or IL-3 treatment and to a 1.7–2.1-fold higher expression than with SLF treatment in three separate experiments.

Levels of junB mRNA were highest at 30 min after factor treatments. SLF induced greater expression of junB mRNA than either GM-CSF or IL-3. Combined treatment of cells with SLF plus either GM-CSF or IL-3 led at 30 min to a 6–10-fold higher expression of junB than with either GM-CSF or IL-3 and to a 1.5–2-fold higher expression than with SLF in three separate experiments. Furthermore, the induction of junB gene expression was maintained at a higher level in cells treated with SLF plus either GM-CSF or IL-3 over the course of the study, as compared with cells treated with each factor alone.

Levels of egr-1 mRNA were greatest at 30–60 min after induction. A higher expression of egr-1 message was induced in SLF- than in GM-CSF- or IL-3-treated cells. Combined

![Fig. 1. Kinetics of immediate-early mRNA induction after treatment of MO7e cells with SLF, GM-CSF, and IL-3. Eighteen hours after factor starvation, MO7e cells were treated with optimal amounts of SLF (50 ng/ml), GM-CSF (500 units/ml), and/or IL-3 (1000 units/ml) for cell proliferation. Cytoplasmic RNAs were isolated at the times indicated. RNA (20 µg) was analyzed on 1.0% agarose gel, and the blots were hybridized with the 32P-labeled c-fos, junB, c-myc, egr-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Similar results were obtained in each of three separate experiments.](image-url)
treatment of cells with SLF plus either GM-CSF or IL-3 led to a 3–10-fold higher expression of egr-1 mRNA at 1 h than with either GM-CSF or IL-3 treatment and to a 1.6–2.9-fold higher expression than with SLF treatment in three separate experiments. The induction of egr-1 gene expression was maintained at a higher level over the course of the study in cells treated with SLF plus either GM-CSF or IL-3, as compared with cells treated with each factor alone.

To examine the effect of different concentrations of growth factors on the induction of immediate-early genes, M07e cells were treated for 30 min with various doses of SLF, GM-CSF, and IL-3 (Fig. 3). Dose-dependent induction of c-fos, junB, egr-1, and c-myc gene expression was observed by these growth factors. The level of c-fos, junB, egr-1, and c-myc gene expression induced by a suboptimal dose of SLF (5 ng/ml) plus an optimal dose of either GM-CSF (500 units/ml) or IL-3 (1000 units/ml) was greater than the additive level of expression of these genes induced by each factor alone (e.g. GM-CSF (500 units/ml) plus SLF (5 ng/ml)) as described in the legend to Fig. 2. The relative expression (R. E.) was calculated as the fold increase over the baseline expression after normalization of the values according to the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA expression, the relative expression was calculated as the fold increase over base line expression (time 0).

SLF plus GM-CSF, as compared with each factor itself was observed in the presence of cycloheximide. There was no significant change in immediate-early gene expression except for c-myc, which was 3-fold reduced in its expression at 4 h without growth factors during cycloheximide treatment itself (data not shown). In the presence of cycloheximide, more prolonged c-fos gene induction by SLF and GM-CSF was observed than that in the absence of cycloheximide (compare Figs. 1 and 4). This suggests that labile or newly synthesized protein(s) are required for quick turn-off of c-fos gene expression.

To further clarify molecular mechanisms leading to greater induction of immediate-early genes by combined treatment of M07e cells with SLF plus GM-CSF than by treatment with each factor alone, it is necessary to ascertain whether regulation is at the transcriptional or posttranscriptional level. Nuclear run-on transcription assays performed in vitro with nuclei isolated from M07e cells before and after stimulation with SLF and/or GM-CSF were used to measure transcription.
Effect of cycloheximide treatment on the induction of immediate-early genes by SLF and GM-CSF. Factor-starved M0\textsuperscript{7}e cells were preincubated with cycloheximide (10 \mu g/ml) for 30 min and then treated with optimal amounts of SLF (50 ng/ml) and/or GM-CSF (500 units/ml). Cytoplasmic RNAs were isolated at the times indicated. RNA (20 pg) was analyzed on 1.0% agarose gel, and similar results were obtained in each of two separate experiments.

Effect of SLF and GM-CSF treatment on the transcription rate of c-fos, junB, and egr-1 genes. Factor-starved M0\textsuperscript{7}e cells were treated with or without SLF (50 ng/ml) and/or GM-CSF (500 units/ml), and cells were collected for the harvest of nuclei at the times indicated. Nuclear run-on transcription assays were later performed, and equal cpm/ml of the purified radioactive RNAs from the respective reactions were hybridized against probes for c-fos, junB, c-myc, egr-1, and a constitutively transcribed positive control gene (\gamma-actin) and against a plasmid negative control (pBR322), which were Southern-blotted onto nylon membrane. Similar results were obtained in each of two independent experiments.

Northern blotting (see Figs. 1–3). Combined treatment of cells with SLF plus GM-CSF led to a similar transcriptional activation of egr-1 gene with SLF treatment at 20 min (Fig. 5) and to a higher rate of transcription than SLF treatment at 30 min (data not shown). Interestingly, a transcription rate of egr-1 gene was maintained at a high level only in cells treated with SLF plus GM-CSF (Fig. 5). This conforms to the kinetics of egr-1 gene induction by SLF and GM-CSF (see Figs. 1 and 2). Activation of egr-1 gene transcription by GM-CSF is consistent with the previous report using murine peritoneal macrophages (40). These data suggest that the greatest induction of c-fos and egr-1 genes by SLF plus GM-CSF treatment is brought about, at least in part, at the level of transcription and that junB gene induction by SLF and GM-CSF is post-transcriptional.

The stability of c-fos, junB, and egr-1 transcripts was studied in cells treated with SLF and/or GM-CSF for 30 min to induce their expression and then actinomycin D for varying intervals to inhibit further transcription (Fig. 6). The half-life of c-fos mRNA as determined by densitometric scanning with normalization to the glyceraldehyde-3-phosphate dehydrogenase signals was 13 min for SLF- or GM-CSF-treated cells and 35 min for SLF- plus GM-CSF-treated cells. Similarly, the calculated half-life of junB mRNA was 15 min for SLF- or GM-CSF-treated cells and 36 min for SLF- plus GM-CSF-treated cells. These results suggest that cooperative stabilization of c-fos and junB messages mediated through SLF and GM-CSF pathways is part of mechanisms for synergistic enhancement of their expression by SLF plus GM-CSF treatment. egr-1 mRNA was stable for 1 h after actinomycin D addition and then quickly destabilized in the subsequent 1 h. There was no difference in the kinetics of decay of the egr-1 message between single factor-treated and SLF- plus GM-CSF-treated cells. Thus, mRNA stabilization does not appear to be responsible for greater induction of egr-1 expression in cells treated with SLF plus GM-CSF than seen with each factor alone. Although we were not able to examine the stability of the egr-1 message in unstimulated cells because of the low level of its constitutive expression, the half-lives of egr-1 mRNA (>1 h) of SLF- and/or GM-CSF-treated M0\textsuperscript{7}e cells are longer than that (13–21 min) of GM-CSF-treated peritoneal macrophages, in which the induction of egr-1 gene is governed mainly by transcriptional activation (40). This implies that the induction of egr-1 gene expression by GM-CSF or SLF may be mediated in part through the stabilization of egr-1 transcript in M0\textsuperscript{7}e cells. Taken together, our data
indicate that synergistic enhancement occurs, at least in part, at the level of transcription and mRNA stabilization for c-fos, at the level of mRNA stabilization for junB, and at the level of transcription for egr-1.

**DISCUSSION**

Binding of growth factors to their receptors results in the transmission of a signal to the nucleus via pre-existing cytosolic effector molecules, where a programmed process of gene expression takes place (reviewed in Ref. 1). A number of growth factors, including GM-CSF, IL-3, and CSF-1 induce immediate-early gene expression (41-44). Members of Fos and Jun families, which together form the AP1 transcription factor complex, have been implicated in the entry of cells into S phase of the cell cycle (11, 12). Egr-1 protein binds to DNA and activates transcription of the target genes, whose products are required for mitogenesis and differentiation (14). c-Myc protein is also known to play a key role for G1 to S and S to G2/M transition triggered by CSF-1 and by epidermal growth factor, respectively (45, 46). Our present data point to growth factor regulation of c-fos, junB, egr-1, and c-myc mRNAs as a component of the regulatory process that governs synergistic cell growth by SLF when used in combination with either GM-CSF or IL-3. We believe that this is the first reported evidence for at least a portion of the mechanistic pathway leading to SLF- plus GM-CSF- or IL-3-induced synergistic cell proliferation.

SLF synergizes with GM-CSF and IL-3 to stimulate cell proliferation of the human factor-dependent cell line, MO7e (32), and these factors induce tyrosine, serine, and threonine phosphorylation in MO7e cells (31). A number of proteins become phosphorylated by these factors and some common signaling pathway have been indicated between SLF and GM-CSF, including to a very limited extent GTPase-activating protein and to greater extent GTPase-activating protein-associated protein p190, Raf-1, and mitogen-activated protein kinase (31). We have observed similar sets of immediate-early genes induced by SLF and GM-CSF, which is consistent with the concept that these factors share some molecules for transducing signals. IL-3 induces a similar, if not identical, pattern of tyrosine phosphorylation (47) and immediate-early gene expression with GM-CSF in MO7e cells, which conforms to the idea that these two cytokine receptors share a similar signal transducing subunit (48, 49). Combined treatment with SLF plus either GM-CSF or IL-3 induced greater expression of c-fos, junB, egr-1, and c-myc genes than each factor alone in the presence or absence of protein synthesis. A higher rate of c-fos and egr-1 gene transcription and greater stability of c-fos and junB transcripts were seen in cells treated with SLF plus GM-CSF than in cells treated with each factor alone. These results suggest that SLF and GM-CSF activate transcription of c-fos and egr-1 genes and stabilize c-fos and junB mRNAs in a cooperative fashion through pre-existing molecule(s), resulting in greater gene expression. Our finding that SLF and GM-CSF regulate c-fos expression by both transcriptional and posttranscriptional mechanisms is similar to regulation of c-fos gene expression by CSF-1 in human monocytes (50).

Two distinct regions are known to be responsible for rapid decay of c-fos transcript. One is located in the protein-coding segment, and the other is an AU-rich segment, which is also found in junB and egr-1 genes, located within the 3'-untranslated region (51). A degradation pathway through an AU-rich segment of the c-fos gene is dependent on RNA synthesis (51). This partly explains why c-fos, junB, and egr-1 mRNAs were more stable in the presence of actinomycin D than in its absence (data not shown). It has been reported that cycloheximide treatment increases the stability of junB transcript induced by 12-O-tetradecanoylphorbol-13-acetate in HL-60 promyelocytic leukemia cells (52), suggesting that junB gene is regulated by a labile protein at the level of posttranscription. It is noteworthy that two growth factors act cooperatively to augment stability of junB and c-fos mRNAs.

Our findings point out that SLF and GM-CSF act on a similar set of immediate-early genes in a different way and that a combination of SLF plus GM-CSF has features other than just the additive effects of each growth factor in the induction of transcription factor genes, which may in turn affect the expression of genes responsible for cell proliferation. Thus, synergism of SLF with either GM-CSF or IL-3 on cell proliferation may, in part, be achieved by reprogramming the level and proportion of serial immediate-early gene expression induced by GM-CSF and IL-3. This model should serve as a guide to further studies evaluating the molecular basis of cytokine stimulation of hematopoietic cell proliferation.
Immediate-early Gene Regulation by SLF, GM-CSF, and IL-3

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