

Stem Cell Factor Enhances Interleukin-3–Dependent Induction of 68-kD Calmodulin-Binding Protein and Thymidine Kinase Activity in NFS-60 Cells

By G. Prem Veer Reddy and Peter J. Quesenberry

Stem cell factor (SCF) is known to act synergistically with other hematopoietic factors in increasing the colony formation of hematopoietic progenitor cells. We have shown that interleukin-3 (IL-3)–dependent proliferation of NFS-60 cells is associated with the induction of a specific calmodulin-binding protein of about 68 kD (CaM-BP68). To evaluate the relationship between proliferative stimulation and the induction of CaM-BP68 by cytokines, we examined whether the increased proliferative potential of NFS-60 cells in response to SCF is reflected in an increased induction of the CaM-BP68. We observed that SCF alone has a limited effect on proliferative stimulation and on the induction of CaM-BP68 in factor-deprived NFS-60 cells. However, when combined with IL-3, granulocyte colony-stimulating factor (G-CSF), or IL-6, it caused a significant increase in cytokine-dependent proliferative stimulation, as well as in the

PROLIFERATION OF hematopoietic progenitor cells is often dependent on the extracellular stimuli involving specific cytokines.^{1–5} These cytokines include the hematopoietic colony stimulating factors (CSFs), interleukin-3 (IL-3, multi-CSF), granulocyte-macrophage CSF (GM-CSF), interleukin-6 (IL-6), granulocyte CSF (G-CSF), and erythropoietin (Epo) belonging to hematopoietin receptor super family,⁶ and macrophage CSF (M-CSF or CSF-1) and stem cell factor (SCF; also known as c-kit-ligand, mast cell growth factor, and steel factor) belonging to the receptor tyrosine kinase family.⁷ While each of these cytokines by themselves are capable of inducing proliferative stimulation, when presented in combination with other cytokines of its own, or of the other family, they show a marked increase in their potential to induce proliferative stimulation of factor-dependent myeloid progenitor cells.^{8–11} As each of the cytokines activate a chain of events specific to its interaction with the cognate receptor, how divergent events activated by multiple cytokines in a combination converge to elicit a common biological response governing proliferative stimulation is not known.

SCF, by binding to its receptor encoded by the c-kit proto-oncogene,¹² enhances the effect of multiple cytokines including GM-CSF, G-CSF, IL-1 β , IL-3, IL-6, IL-7, IL-9, and IL-12 in inducing proliferative stimulation of factor-dependent multipotent hematopoietic progenitor cells.^{1–3,11,13–19} This is accompanied by tyrosine phosphorylation of a number of proteins due to tyrosine kinase activity that is intrinsic to SCF receptor.^{20,21} These proteins include c-kit itself (by autophosphorylation), phospholipase C- γ (PLC- γ), Raf-1, MAP kinase, and GTPase-activating protein (GAP).^{20–25} Some of these same proteins are also being phosphorylated and/or activated by hematopoietic growth factors such as IL-3 and GM-CSF, the receptors of which belong to a related hematopoietin receptor family that has no intrinsic tyrosine kinase activity.^{24,25} These observations highlight not only the importance of tyrosine phosphorylation events in cytokine receptor interactions, but also point to the convergent nature of signal transduction pathways of two different receptor families. However the quantitative analysis of such phosphoproteins

induction of CaM-BP68. Furthermore, an increase in IL-3–dependent induction of CaM-BP68 in the presence of SCF coincided with a corresponding increase in thymidine kinase activity, whose expression is linked to G₁/S transition of the cells. At low concentrations SCF caused a synergistic increase in IL-3–dependent induction of both CaM-BP68 and thymidine kinase activity. In contrast to the changes in CaM-BP68 and thymidine kinase activity, no significant changes in DNA polymerase α were observed in factor-deprived NFS-60 cells in response to IL-3 and/or SCF. These observations suggest an increased expression of CaM-BP68 and thymidine kinase are associated with the synergistic effect of SCF on factor-dependent proliferation of hematopoietic progenitor cells.

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generated by the individual or combined cytokine action has failed to provide the basis for the synergistic effect of SCF when combined with other cytokines, such as IL-3 or GM-CSF, on proliferative stimulation of myeloid progenitor cells.^{22–25}

In an effort to understand the molecular mechanism of cytokine action in inducing proliferative stimulation of a variety of hematopoietic progenitor cells, we have identified a specific calmodulin-binding protein of 68 kD, termed CaM-BP68.²⁶ This protein has been shown to modulate in response to specific cytokines; this modulation correlates temporally with the induction of proliferation. Its induction in follicular dendritic cells (FDC)-P1 cells coincides with their factor (IL-3)–dependent progression from G₁ to S phase.²⁶ Purified CaM-BP68 is shown to stimulate DNA synthesis in permeabilized quiescent FDC-P1 cells.²⁷ Most recently, CaM-BP68 has been found tightly associated with DNA polymerase α -primase complex that is a subcomponent of a 21S mega complex capable of replicating SV40 DNA in vitro.²⁸ Thus, CaM-BP68 represents a novel protein that links cytokine generated signals to the enzymes of DNA synthesis in the nuclei. Based on these observations, we examined whether the enhancing effect of SCF on IL-3–dependent proliferation of hematopoietic progenitor cells is related to the modulation of CaM-BP68.

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MATERIALS AND METHODS

Cell culture. The IL-3-dependent myeloid leukemic cell line, NFS-60²⁹ (obtained from Dr Donna Rennick, DNAX Corp, Palo Alto, CA), was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 5% fetal calf serum (FCS) (purchased from Hyclone Laboratories Inc, Logan, UT), 10% WEHI-3 cell-conditioned medium (WEHI-3 cm), 50 μ mol/L mercaptoethanol, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified incubator with 5% CO₂. Experiments were performed with these cells when they were in their early passages and were tested to be mycoplasma-free.

Cytokine-deprivation and readdition. NFS-60 cells grown to a density of about 1×10^6 cells/mL were harvested by centrifugation at 500g for 5 minutes at room temperature and were washed once in culture medium containing FCS, but no WEHI-3 cm or cytokines. Cells were then suspended to the density of 1×10^6 cells/mL in medium without WEHI-3 cm and incubated in humidified incubator at 37°C for 16 hours. At the end of the incubation, cell viability was determined by their ability to exclude Trypan Blue. Individual cytokines or WEHI-3cm was then added to the medium of the cultures that contained more than 90% viable cells. Ten to 12 hours following the readdition of cytokines or WEHI-3 cm cells were analyzed for their ability to incorporate ³H-thymidine, and for the presence of CaM-BP68, thymidine kinase, and DNA polymerase activities in their lysate.

Measurement of ³H-thymidine incorporation into DNA. One-milliliter aliquots of exponentially growing, cytokine-deprived, and cytokine-read cells at a density of about 1×10^6 cells/mL were incubated with 5 μ Ci/mL of ³H-thymidine (purchased from ICN Biomedicals, Inc, Costa Mesa, CA) at 37°C in a humidified incubator for 60 minutes. Cell counting in these studies was performed by using Coulter Counter Z_i Model (Coulter Electronics Inc, Hialeah, FL). Cells were then subjected to trichloroacetic acid (TCA) precipitation by treating with 10% TCA for 30 minutes on ice. Acid precipitate was washed once with 2 mL ice-cold deionized distilled H₂O and twice with 2 mL ice-cold methanol. Recovery of acid precipitate and its wash with ddH₂O was achieved by centrifugation at 5,000 rpm and subsequent two methanol washes by centrifugation at 10,000 rpm for 10 minutes each. The precipitate recovered following a second methanol wash was dissolved in 0.5 mL of 0.4 mol/L NaOH and a 50- μ L aliquot of the sample combined with 5 mL of Sigma-Fluor (Sigma Chemical Co, St Louis, MO) was counted to determine ³H-TdR incorporation into acid-insoluble material.

Preparation of cell lysate. Cells harvested by centrifugation were washed once with buffer A (35 mmol/L HEPES [7.4], 150 mmol/L sucrose, 80 mmol/L KCl, 5 mmol/L potassium phosphate [7.4], 5 mmol/L MgCl₂, 8 mmol/L dithiothreitol (DTT), and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)). Cells were then suspended in buffer A at a density of 2×10^7 cells/mL and homogenized in a top-driven Wheaton homogenizer (Wheaton Instruments, Midville, NJ) until about 90% of the cells were stained with Trypan Blue. The supernatant obtained by centrifugation at 700g and 4°C for 10 minutes was considered as the cytoplasmic fraction in which thymidine kinase and DNA polymerase activities were measured. The pellet containing nuclei was suspended in buffer A to the same density as cells and sonicated using a Branson Sonifier 250, equipped with micro-tip (Branson Sonic Power Co, Danbury, CT), at an output setting of 1.5 to 2 and a duty cycle of 20% for 30 to 40 pulses. The nuclear homogenate was then cleared by centrifugation at 5,000 rpm and 4°C for 10 minutes in a RC-5B Sorvall centrifuge equipped with a SS-34 rotor, and the supernatant was treated as the nuclear fraction in which CaM-BP68 content was determined.

CaM-BP68 detection. CaM-BP68 in nuclear fractions was de-

tected essentially as described elsewhere.^{26,27} Either 20 or 50 μ g of protein in the nuclear fractions, depending on the degree of sensitivity required to estimate relative levels of CaM-BP68 in nuclear lysate, was subjected to denaturing polyacrylamide gel electrophoresis and the proteins resolved on the gels were transferred to nitrocellulose filters. CaM-BPs on the filters were then identified by using biotinylated CaM (purchased from Biomedical Technologies, Inc, Stoughton, MA) and alkaline phosphatase-conjugated avidin (purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN) as described by Billingsley et al.³⁰ Prestained molecular weight markers (purchased from Bethesda Research Laboratories, Bethesda, MD) were used to identify 68 kD CaM-BP on the filters.

Densitometric analysis of CaM-BP68 bands on the filters. To obtain an estimate of relative levels of CaM-BP68 in the cells exposed to various concentrations of cytokines, the CaM-BP bands on the filters were scanned by using Apple OneScanner connected to Apple Macintosh IIsi microcomputer (Apple Computer, Cupertino, CA) and Ofoto flatbed scanner software (Light Source Computer Image; distributed by Apple Computer). The digital images thus captured were analyzed using ScanAnalysis software package (Bio-Soft, Cambridge, UK). The relative level of CaM-BP68 is then expressed in arbitrary units (AU) that is derived by dividing the density of the CaM-BP68 band with the band density of an unknown CaM-BP of a higher molecular weight that showed no significant changes in response to cytokine-deprivation or to the altered growth conditions. Expression of CaM-BP68 levels in relation to the relatively stable protein, termed "protein X", in individual samples served as an internal control to correct for any minor sampling errors that may have occurred during sample preparation and/or polyacrylamide gel electrophoresis of individual samples. Thus, the CaM-BP68 levels in individual samples is expressed as AU = (density of CaM-BP68 band - background density) \div (density of protein X band - background density), where background density is obtained by scanning a corresponding segment of individual sample lanes on the filter that are devoid of any visible bands.

Thymidine kinase and DNA polymerase assays. Thymidine kinase and DNA polymerase- α activity in individual cytoplasmic fractions was determined as described by Subramanyam et al.³¹

Protein estimation. Protein content in individual fractions was determined according to the method of Lowry et al.³²

RESULTS

SCF augments IL-3- and G-CSF-dependent induction of CaM-BP68 and ³H-thymidine incorporation in factor-deprived NFS-60 cells. In an approach to understand the molecular basis for SCF action in augmenting cytokine-dependent proliferative stimulation of hematopoietic progenitor cells, we have examined the effect of SCF on the induction of CaM-BP68 in NFS-60 cells. As reported earlier, cytokine-deprivation of NFS-60 cells leads to a significant decrease in their nuclear CaM-BP68 levels²⁶ (Fig 1). This decrease in CaM-BP68 is reversed to some extent by readdition of IL-3, G-CSF, IL-6, or SCF alone. However, when SCF is combined with IL-3 or G-CSF, the induction of CaM-BP68 level increased to a level greater than that observed in cells growing continually in the presence of WEHI-3 cm. Furthermore, it was observed from densitometry analysis (Fig 2) that SCF in combination with IL-3, IL-6, or G-CSF resulted in an additive effect on the induction of CaM-BP68 and resulted in CaM-BP68 levels that are comparable to those observed in log-growing cultures (Fig 2). These changes in

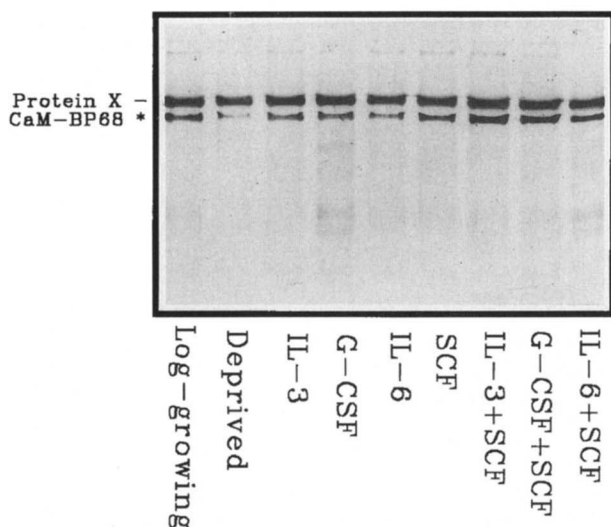


Fig 1. Effect of IL-3, G-CSF, IL-6, and SCF on the induction of CaM-BP68 in factor-deprived NFS-60 cells. Exponentially growing NFS-60 cells at a density of about 1×10^6 cells/mL were deprived of cytokines for 20 hours. Cytokine-deprived cells were then treated with IL-3 (100 U/mL, GIBCO), hG-CSF (1,000 U/mL, Amgen), IL-6 (20 ng/mL, Genetics Institute), SCF (200 ng/mL, Amgen) or with SCF in combination with IL-3, hG-CSF, or IL-6 at the same concentrations for 12 hours. Nuclear lysates were prepared, and the CaM-BPs in the lysate fractions were determined as described in Materials and Methods.

CaM-BP68 levels showed a direct correlation to the changes in the ability of NFS-60 cells to incorporate ^3H -thymidine into their DNA in response to each of the cytokine conditions (Fig 2).

SCF alone is ineffective in completely reversing the effects of cytokine-deprivation on NFS-60 cells. We have examined whether the induction of CaM-BP68 could be influenced by the increasing concentrations of SCF alone. We observed a gradual increase in the induction of CaM-BP68 with increasing SCF concentration from 5 to 25 ng/mL (Fig 3). Although this increase in CaM-BP68 levels in response to SCF seems to be twofold to threefold lower than that observed with IL-3 at a similar concentration (Fig 3), it corresponded closely with the cellular ability to incorporate ^3H -thymidine (Fig 4). Furthermore, it is intriguing to note that the ability of factor-deprived cells to induce CaM-BP68 could not be enhanced any further than that observed with 25 ng/mL of SCF by increasing SCF concentration even up to 1,000 ng/mL (Fig 5). Thus, while SCF alone is capable of inducing CaM-BP68 in proportion to its ability to stimulate ^3H -thymidine incorporation in factor-deprived cells, the overall induction of both these processes by SCF alone remained relatively lower than that observed with IL-3 alone or with WEHI-3 cm in factor-deprived NFS-60 cells.

Low concentrations of SCF enhance the effectiveness of IL-3 in inducing CaM-BP68 and ^3H -thymidine incorporation. As shown in Fig 1, when relatively high concentrations of SCF (200 ng/mL) and IL-3 (100 U/mL) were combined together, there was an additive effect of these cytokines on the induction of both the CaM-BP68 and ^3H -

thymidine incorporation. In an effort to understand the synergistic nature of SCF in hematopoietic progenitor cell proliferation, we tested the effect of low concentrations (1 to 2 ng/mL) of SCF on IL-3-dependent induction of CaM-BP68 and ^3H -thymidine incorporation in NFS-60 cells. Such low concentrations of SCF alone caused no significant induction of either CaM-BP68 or ^3H -thymidine incorporation in factor-deprived cells (Fig 3). However, such low concentrations of SCF when combined with IL-3 caused a 100% increase in the effectiveness of various concentrations of IL-3 to induce CaM-BP68 (Fig 6). Furthermore, the enhancing effect of SCF on IL-3-dependent induction of CaM-BP68 (expressed quantitatively in Fig 6B) corresponded with an increase in the rate of ^3H -thymidine incorporation into DNA (Fig 7).

SCF-mediated changes in CaM-BP68 coincide with the changes in thymidine kinase activity in factor-deprived NFS-60 cells. To further evaluate the significance of the enhancing effect of SCF on IL-3-dependent induction of CaM-BP68 and the stimulation of ^3H -thymidine incorporation, we examined the changes in the expression of thymidine kinase activity in response to SCF and IL-3. Thymidine kinase expression in a variety of mammalian cells is linked to their ability to commit for proliferation and to traverse through cell cycle from G_1 to S phase.³³ As in the case of CaM-

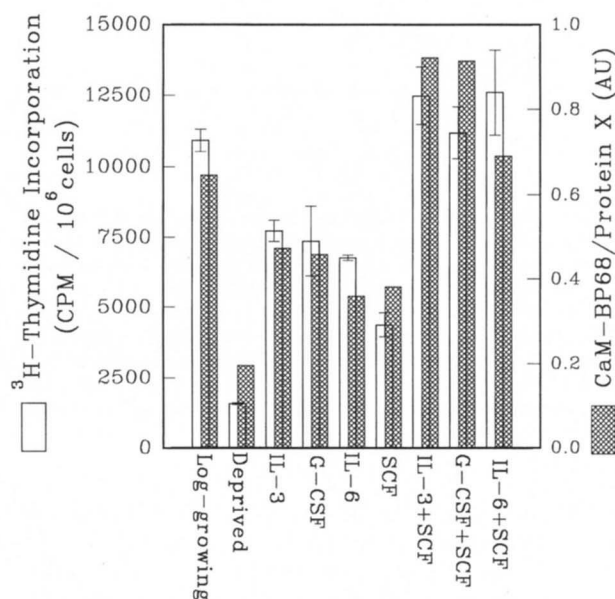


Fig 2. Relationship between cytokine-mediated CaM-BP68 induction and the cellular ability to incorporate ^3H -thymidine. NFS-60 cells were deprived of cytokines and then treated with IL-3, hG-CSF, IL-6, or SCF as described in the legend to Fig 1. One-milliliter duplicate aliquots of cells from each of the cytokine-treated conditions were incubated with ^3H -thymidine, and the radioactivity incorporated into DNA was determined (open bars). Experimental procedures were as described in Materials and Methods. Error bars represent the mean of duplicate determinations in each of two experiments. Relative amounts (AU) of CaM-BP68 (shaded bar) was determined by the densitometry analysis of the 68 kD band (CaM-BP68) and the 76 kD band (protein X) in each lane on the original filter of Fig 1 as described in Materials and Methods.

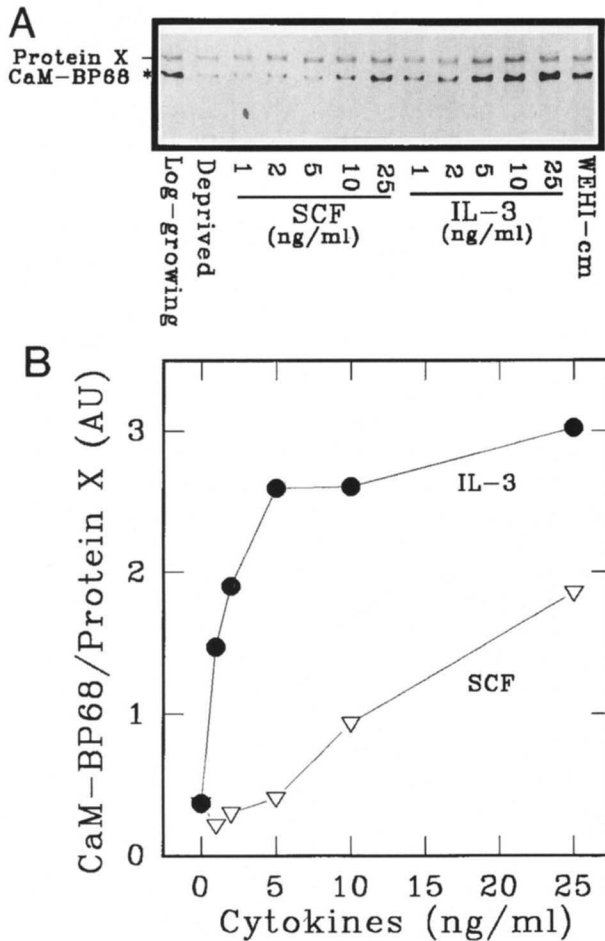


Fig 3. Effect of increasing concentrations of SCF and IL-3 on CaM-BP68 induction in factor-deprived cells. Log-growing NFS-60 cells were deprived for cytokines for 20 hours and then incubated with the increasing concentrations of SCF or IL-3. Ten hours following cytokine treatment, nuclear lysates were prepared and subjected to CaM-BP68 analysis for the visualization of the protein bands on the filters (A) and for the estimation of relative amounts (AU) of CaM-BP68 (B). Experimental procedures for the lysate preparation, CaM-BP68 detection and the densitometry analysis are as described in Materials and Methods.

BP68, thymidine kinase activity decreased significantly in the cells deprived of either WEHI-3cm or the cytokines required for their proliferation (Fig 8). Readdition of IL-3 alone is capable of inducing the expression of thymidine kinase activity in factor-deprived NFS-60 cells (Fig 8). The induction of thymidine kinase activity also showed a linear relationship to IL-3 concentrations ranging from 1 to 10 ng/mL. At 25 ng/mL and higher concentrations of IL-3, the level of thymidine kinase activity remained relatively unchanged (Fig 8 and data not shown). However, when 2 or 5 ng/mL of SCF is combined with IL-3, there is a significant increase in the overall expression of thymidine kinase activity (Fig 8) as compared with that observed in the presence of IL-3 alone. SCF alone, at 2 to 5 ng/mL, had no significant effect on the induction of thymidine kinase activity and at

25 ng/mL, its induction was proportionate to the cellular ability to incorporate ^3H -thymidine (data not shown). These changes in thymidine kinase activity in response to SCF and IL-3 are comparable to those observed in the case of CaM-BP68 under similar experimental conditions (compare Figs 6 and 8). By contrast, DNA polymerase α activity, that exhibits little or no change in response to factor-deprivation, remained relatively unchanged when factor-deprived cells are treated with IL-3 alone or with IL-3 and SCF (Fig 8). This is not surprising considering that DNA polymerase α activity in the cells is relatively more stable than thymidine kinase activity and that its nuclear localization, rather than the increase in its overall activity, as the cells enter into S phase seems to be responsible for cellular ability to proliferate.^{34,35}

DISCUSSION

In an attempt to understand the signaling events that are specifically related to the cytokine-dependent proliferative stimulation of hematopoietic progenitor cells, we have examined the ability of cytokines to induce CaM-BP68 and thymidine kinase activity, whose expression and nuclear organization are linked to the cellular commitment to DNA synthesis in NFS-60 cells. These studies demonstrate that individual cytokines, namely, IL-3, G-CSF, IL-6, and SCF induce CaM-

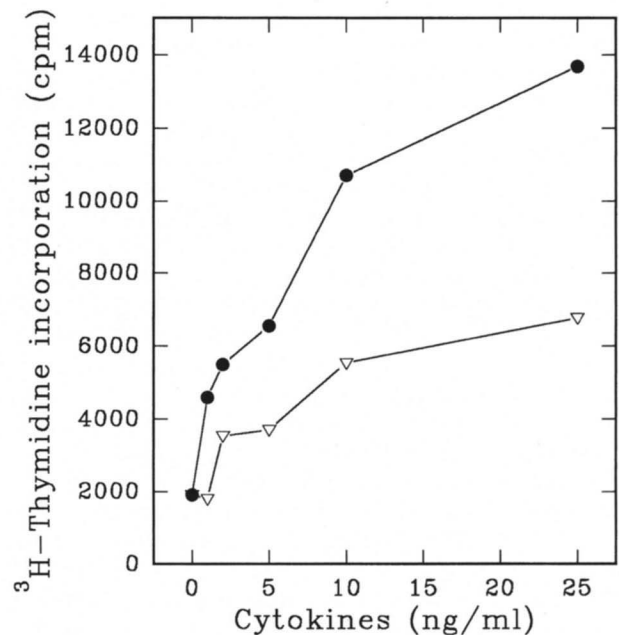


Fig 4. Effect of increasing concentrations of SCF and IL-3 on ^3H -thymidine incorporation. Experimental procedures for cytokine-deprivation and treatment are as described in the legend to Fig 3 except that duplicate aliquots of 1-mL cultures were withdrawn from individual cultures before the preparation of nuclear lysates and incubated with ^3H -thymidine. The amount of radioactivity incorporated into DNA of individual cell cultures was then determined as described in Materials and Methods. The values are expressed as the average of duplicate determinations. There was less than 10% variation between duplicate determinations.

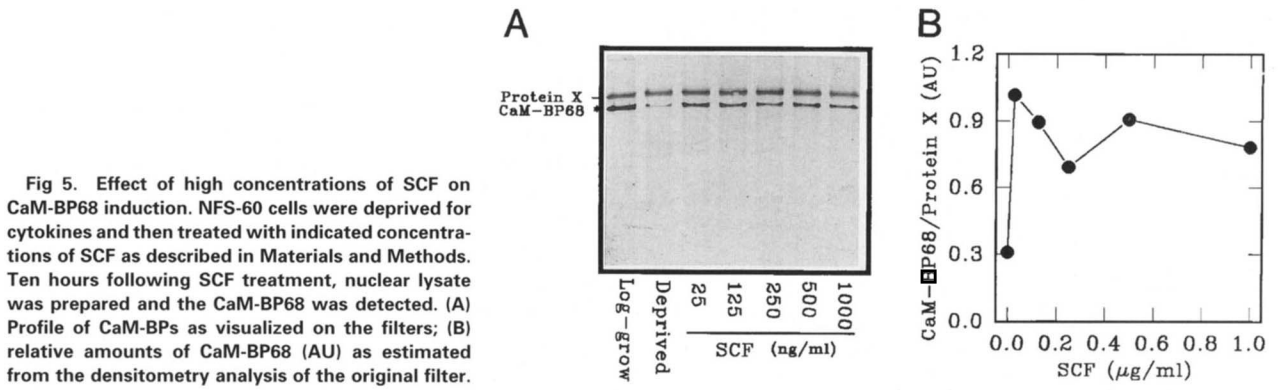


Fig 5. Effect of high concentrations of SCF on CaM-BP68 induction. NFS-60 cells were deprived for cytokines and then treated with indicated concentrations of SCF as described in Materials and Methods. Ten hours following SCF treatment, nuclear lysate was prepared and the CaM-BP68 was detected. (A) Profile of CaM-BPs as visualized on the filters; (B) relative amounts of CaM-BP68 (AU) as estimated from the densitometry analysis of the original filter.

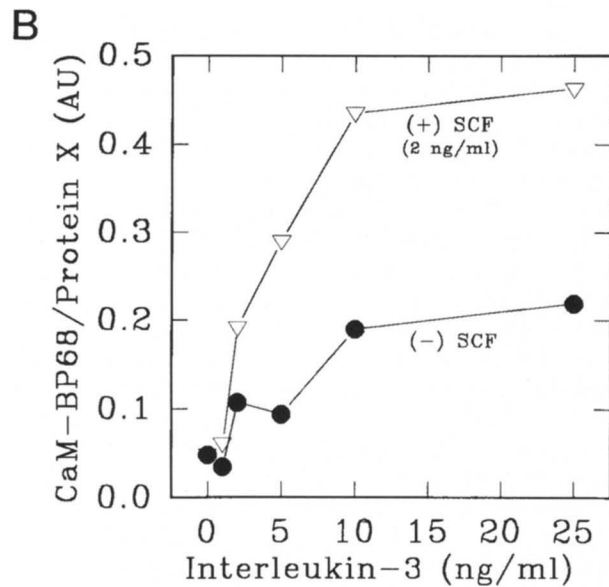
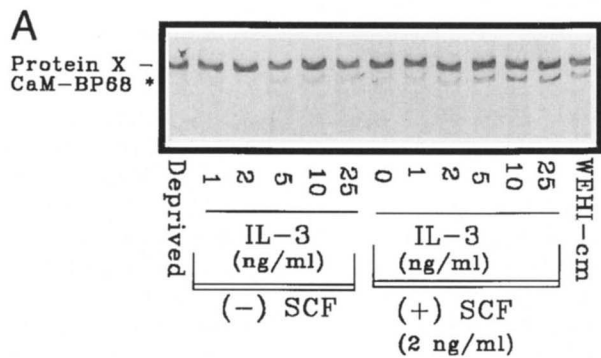


Fig 6. Effect of low concentrations of SCF on IL-3-mediated induction of CaM-BP68. Log-growing NFS-60 cells were deprived for cytokines for 20 hours and then treated with increasing concentration of IL-3 in the presence (∇) or in the absence (\bullet) of 2 $\mu\text{g/ml}$ of SCF. Ten hours following the cytokine treatment, nuclear lysates were prepared and CaM-BP68 levels determined. (A) Profile of CaM-BPs as visualized on the filters in CaM-binding assay and (B) relative amount of CaM-BP68 (AU) determined by the densitometry analysis as described in Materials and Methods.

BP68 in proportion to their ability to cause proliferative stimulation in factor-deprived NFS-60 cells. This observation is consistent with the earlier reports that, in factor-dependent hematopoietic progenitor cells, induction and nuclear organization of CaM-BP68 by the cytokines is associated with their ability to induce proliferative stimulation.²⁶ Furthermore, SCF in combination with IL-3, G-CSF, or IL-6 has an additive effect on both the CaM-BP68 induction and the ³H-thymidine incorporation (Figs 1 and 2). This demonstrates that in NFS-60 cells, SCF combination with other cytokines causes an enhanced proliferative stimulation and that such stimulation is accompanied by a concomitant increase in the induction of CaM-BP68.

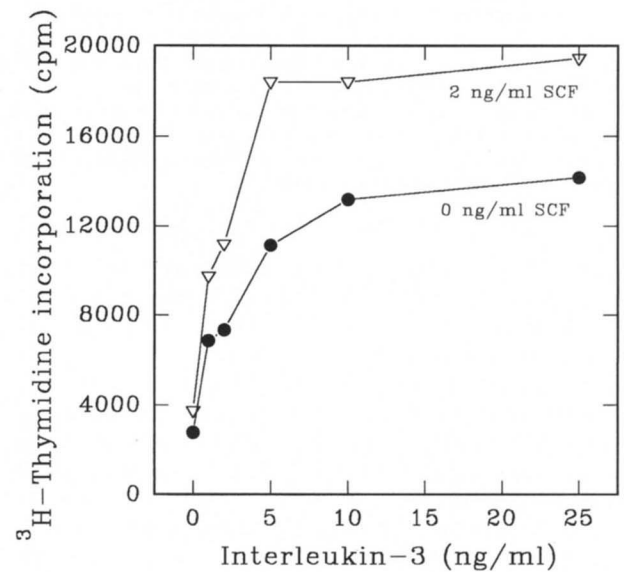


Fig 7. Effect of SCF on IL-3-dependent induction of ³H-thymidine incorporation in factor-deprived cells. NFS-60 cells grown to a density of 1×10^6 cells/mL were deprived of cytokines for 20 hours and then treated with increasing concentrations of IL-3 in the presence or absence of SCF (2 ng/mL). Ten hours following cytokine stimulation, duplicate aliquots of cells were incubated with ³H-thymidine and the radioactivity incorporated into acid-insoluble material determined as described in Materials and Methods. The values are expressed as the average of duplicate determinations. There was less than 10% variation between duplicate determinations.

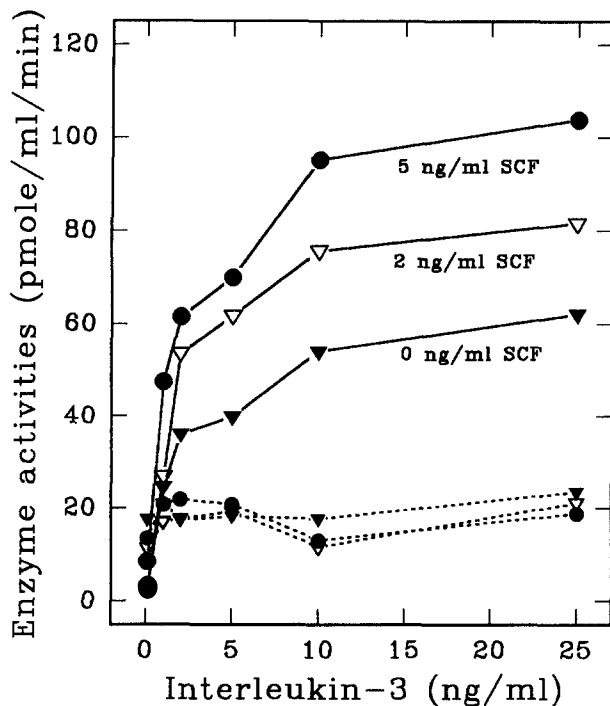


Fig 8. Effect of low concentrations of SCF on IL-3-mediated induction of thymidine kinase and DNA polymerase α activities in cytokine-deprived NFS-60 cells. Log-growing NFS-60 cells were deprived of cytokines for 20 hours and exposed to increasing concentrations of IL-3 in the presence (∇ 2 ng/mL; \bullet , 5 ng/mL) or the absence (\blacktriangledown) of SCF. Ten hours following cytokine treatment, cells were harvested and the cytoplasmic fraction prepared as described in Materials and Methods. Duplicate aliquots from individual fractions were assayed for thymidine kinase and DNA polymerase α activities as described in Materials and Methods. Values are expressed as the average of duplicate determinations. There was less than 5% variation between duplicate determinations of thymidine kinase or DNA polymerase α activities in individual fractions. Solid line, thymidine kinase activity; dashed line, DNA polymerase α activity.

IL-3 seems to be twofold-to-threefold more effective than SCF in inducing both CaM-BP68 and cellular proliferation as indicated by the rate of ^3H -thymidine incorporation in NFS-60 cells (Figs 3 and 4). This observation is consistent with a greater potency of IL-3 or GM-CSF alone than that observed with rhSCF to stimulate the formation of colonies by $\text{CD}34^+$ and $\text{CD}34^+/\text{Lin}^-$ cells³⁶ and to induce ^3H -thymidine incorporation in M07 cells.^{37,38} At low concentrations (less than 5 ng/mL), SCF alone has little or no effect on the induction of CaM-BP68 or on the ^3H -thymidine incorporation (Figs 3 and 4). However, such low concentrations of SCF when combined with increasing concentrations of IL-3 caused a significant increase in these activities, as compared with those observed with the corresponding concentrations of IL-3 alone (Figs 6 and 7). Thus, SCF augments or synergizes IL-3-mediated events associated with the proliferative stimulation of NFS-60 cells. These events include the induction of CaM-BP68 and thymidine kinase activity, a marker for cellular ability to transit from G_1 to S phase³³ and concomitant incorporation of ^3H -thymidine.

During quantitative analysis of CaM-BP68 in the samples from different experiments, it is noticed that the AU values, derived by dividing the density of CaM-BP68 band with that of the protein X band, have varied from experiment to experiment. Based on the densitometry analysis, we believe that the variation in the CaM-BP68/protein X ratio from experiment to experiment is due to the differences in the level of protein X band staining on the filters. For instance, when staining of the protein X band in Fig 3A was relatively lower than that seen in Figs 1, 5A, or 6A, the AU values expressing CaM-BP68 levels in that experiment (Fig 3B) were higher than those observed in the other experiments (Figs 2, 5B, or 6B). Because of these variations in AU values from experiment to experiment, the CaM-BP68 levels were compared only between multiple samples within an individual experiment and not between the samples from two different experiments. Despite such variation in AU values, the interpretive results are highly reproducible and are consistent between many experiments.

Synergistic effect of SCF on IL-3-dependent induction of some of the enzymes/proteins associated with DNA replication in these studies suggests that the postreceptor signals activated by SCF are being combined at a downstream point of signal transduction pathways with the signals generated by IL-3 to produce an enhanced expression and/or activation of genes or gene products that participate in DNA synthesis. Such an increased expression of enzymes/proteins responsible for DNA synthesis may contribute to the enhanced rate of hematopoietic progenitor cell proliferation. This possibility is consistent with recent reports that two major signalling pathways, one activated by *Ras* and the other by Janus family of tyrosine kinases (Jak-STAT pathway) are combined at MAP-kinase, which, in turn, is involved in transcriptional regulation of many proliferation-associated proteins.³⁹ Alternatively, it may also be possible that SCF acting as competence factor may effectively induce cytokine-deprived quiescent cells into proliferative cycle. This would allow the entry of more of the quiescent cells into proliferative cycle by SCF than can be achieved with IL-3 alone. Such an increased entry of quiescent cells into proliferative cycle may have contributed to the increased expression of the enzymes/proteins of DNA replication in SCF stimulated cultures. Such a possibility of SCF acting as a competence factor and GM-CSF or IL-3 acting as a progression factor in proliferative stimulation of hematopoietic progenitor cells is also suggested from the studies with M07 cells.³⁷

Regardless of the specific mechanism, our findings demonstrate that the enzymes/proteins, whose activities are closely related to DNA synthesis are being modulated in a direct response to SCF and that SCF enhances IL-3-dependent expression of CaM-BP68 and thymidine kinase activity in proportion to its effect on proliferative stimulation of hematopoietic progenitor cells.

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