Transforming growth factor-β (TGF-β) has potent positive or negative regulatory properties on hematopoiesis depending on the responding cell type and the context of other regulators present. The observation that TGF-β acts as an inhibitor of granulocyte-macrophage colony-stimulating factor (GM-CSF)-, interleukin-3 (IL-3)- and erythropoietin (Epo)-stimulated proliferation of colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM) and burst-forming unit-erythroid (BFU-E), while not affecting or even stimulating colony-forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GM) and CFU-erythroid (CFU-E) colony formation has led to the hypothesis that TGF-β acts as a selective negative regulator of early hematopoiesis sparing the more committed progenitor cell pool. It has also been shown that growth factor-stimulated proliferation of acute myelogenous leukemia (AML) cells is inhibitable by TGF-β. As in normal hematopoiesis, the TGF-β response of leukemic myelogenous progenitor cells appeared to be heterologous and resistance to TGF-β inhibition was suggested to be indicative for leukemia progression. The mechanisms by which TGF-β inhibits hematopoiesis are not completely understood. However, it has been shown that suppression of cell growth by TGF-β relies on its reversible inhibition of cell entry into cycle, implicating the phosphorylation status of pRb and c-myc gene transcription.
myelogenous leukemia cells requires functional c-jun/AP-1 and that the transcriptional activation of c-jun/AP-1 is preceded by binding of NF-jun to its recognition sequence at position -140 to -131 of the c-jun promoter. We show here that SCF activates nuclear translocation of NF-jun, stimulates binding of NF-jun to its specific binding site, and thereby transcriptionally activates c-jun expression. Although TGF-β failed to interfere with the nuclear translocation of NF-jun on SCF, it almost completely abolished NF-jun binding activity to the c-jun promoter and abrogated c-jun gene transcription.

MATERIALS AND METHODS

Recombinant cytokines. Recombinant human (rh) SCF (Lot 500-1) was obtained through Amgen Corp (Thousand Oaks, CA), rhTGF-β1 (specific activity, 2 × 10^11 U/mg of protein) was purchased from British Bio-Technology Ltd (Abingdon, UK), rhGM-CSF and rhIL-3 were kindly provided by Immunex (Seattle, WA), P. Angel, Institut für Genetik, Karlsruhe, Germany, and 0.8-kb BamHI/Pst 1 α-actin fragment in pBR 322 (kindly provided by Dr P. J, Schwarz, Baylor College of Medicine, Houston, TX), and the 1.25-kb Pst II fragment of rat GAPDH in pBR322.

Cell culture and proliferation assay. GF-D8 cells (kindly provided by Dr A. Rambaldi, Istituto Di Ricerche Farmacologiche "Ma- rio Negri," Bergamo, Italy) and Mo7 (DSM, Braunschweig, Germany) were maintained in continuous culture in standard culture medium (SCM; composed of RPMI 1640 medium supplemented with 10% low-endotoxin fetal calf serum [FCS; Hazelton, Vienna, UT], 1% penicillin/streptomycin, and 2 mmol/L L-glutamine [Serva, Heidelberg, Germany]) in the presence of rhGM-CSF (50 ng/mL) or rhIL-3 (5 ng/mL), respectively. Before assay, GF-D8 or Mo7 cells were factor- and serum-deprived over a period of 18 hours and then resuspended in serum-free SCM (SFSCM) without rhGM-CSF added, as previously described. Experimental cultures were always performed in SFSCM without rhGM-CSF in the presence or absence of rhSCF (10 ng/mL) and/or rhTGF-β1 (10 ng/mL), unless otherwise indicated. The initial cell density in Northern blot analyses, transcriptional run-on assays, and immunofluorescence analyses was 1 × 10^6 cells/mL and 5 × 10^5 cells/mL, respectively, for proliferation assays. Cultures were performed either in 200-μL culture flasks or in 96-well round-bottom plates (Greiner, Nütingen, Germany). For proliferation analyses, rhSCF-stimulated GF-D8 or Mo7 cells were cultured for 48 hours with 15 kBq tritiated thymidine (3H-Tdr; specific activity, 2 kBq/mmol) being present for the last 6 hours, followed by cell harvest and counting in a liquid scintillation counter. In some cultures, a neutralizing anti–TGF-β1 antisera was present at a concentration of 10 μg/mL.

Detection of TGF-β1 binding proteins. Detection of TGF-β1 binding proteins was performed as previously described. Briefly, cells were pelleted and lysed in cold binding buffer (RPMI 1640 containing 0.1% bovine serum albumin [BSA] and 25 mmol/L 4-(2-hydroxyethyl)-1-1 piperazine-ethanesulfonic acid, pH 7.4) with 50 mmol/L 125I-labeled TGF-β1 (Amersham Buchger, Braunschweig, Germany; specific activity, 4.56 × 10^8 cpm/ng) in the presence or absence of fivefold excess unlabeled TGF-β1 at 4°C. Cells were then resuspended in 100 μg/mL disuccinimidyl suberate (Pierce Chemical, Rockford, IL) to cross-link TGF-β1 to cell surface binding proteins. Membrane proteins were washed in 250 mmol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.4) and lysed in the presence of protease inhibitors. The supernatant was clarified by centrifugation and aliquots representing equal cell numbers were separated by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography.

Cell cycle analysis. Cell cycle analysis was performed on propidium iodide-stained nuclei as previously described. Briefly, cells (1 × 10^6) were harvested at the time points indicated, washed in ice-cold phosphate-buffered saline (PBS), fixed by the addition of 100% ethanol, and incubated for 30 minutes on ice. The cell pellet was washed and resuspended in 500 μL H2O containing RNase A (40 μg/mL) for 30 minutes at room temperature. Thereafter, 500 μL of solution A (consisting of 0.1% vol/vol Triton X [Sigma, München, Germany] and 0.1 mmol/L EDTA) and propidium iodide to a final concentration of 15 μg/mL were added.

Indirect immunofluorescence analysis. SCF-surface receptor expression was assessed by indirect immunofluorescence and flow cytometry, as described before. In brief, cells were incubated with the YB5.B8 MoAb for 30 minutes at 4°C, washed twice in PBS, and resuspended in fluorescein isothiocyanate (FITC)-labeled sheep F(ab’)2 antimouse IgG and IgM antibody for another 30 minutes. Reactivity of YB5.B8 was determined by flow cytometry (FACStar; Becton Dickinson). Changes in expression of surface antigens were quantified by calculating mean fluorescence intensity (MFI) and the percentage of reactive cells. The percentage of positive cells was calculated with the arbitrary 1% cutoff channel position of the negative control (nonbinding and isotype identical MoAb).

Isolation of total cellular RNA. Northern blot analysis, and nuclear run-on transcription assay. Medium- or SCF-treated cells were harvested after the appropriate culture time as indicated. Cells were resuspended in guanidinium isothiocyanate (Sigma) and extracted with an equal volume of acetone/EDTA-equilibrated phenol (60°C for 25 minutes with frequent vortexing). The aqueous phase was recovered after centrifugation and extracted once with an equal volume of phenol/chloroform and twice with chloroform. The resulting RNA was precipitated overnight at -20°C with 2.5 vol of ethanol. The total RNA from each sample was then electrophoresed on a 1% agarose gel containing 20 mmol/L sodium borate, pH 8.3, 0.5 mmol/L EDTA, and 3% formaldehyde. The RNA was transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany) in 10× SSC (1.5 mol/L sodium chloride and 150 mmol/L sodium citrate) using capillary blotting overnight. The blots were baked and prehybridized at 65°C in 7% SDS, 10× Denhardt’s (1× Denhardt’s is 0.2% Ficoll, 0.2% BSA, and 0.2% polyvinyl pyrrolidone), 5× SSC, and 20 mmol/L salmon sperm DNA (Sigma). Probes were radiolabeled by random priming with α[32P]-dCTP (6000 Ci/mmol; Amersham-Buchger). The blots were washed at 55°C in 1% SDS/1× SSC and were autoradiographed with a Kodak X-omat film (Eastman Kodak, Rochester, NY) at -70°C with an intensifying screen. Autoradiographs were scanned using the LKB Ultra Scan XL laser densitometer and analyzed using the LKB Gel Scan XL software package (Pharmacia, LKB Biotechnology, Uppsala, Sweden).

For nuclear run-on transcription assays, cells (10^5) were lysed in RSB (10 mmol/L Tris-HCl, 5 mmol/L KCl, 3 mmol/L MgCl2 containing 0.5% Nonidet P 40 (Sigma) and were washed once in ice-cold PBS. Nuclei were incubated at 26°C in 15% glycerol, 70 mmol/L KCl, 2.5 mmol/L MgCl2, 10 mmol/L EDTA, 4 mmol/L levels each of ATP, CTP, and GTP, 2 mmol/L UTP, 0.5 mmol/L L-dithiothreitol (DTT), 60 U/mL RNasin (Boehringer Mannheim, Mannheim, Germany), in the presence of 100 μCi of α[32P]-UTP (3000 Ci/mmol; Amersham-Buchger) for 30 minutes. The mixture was digested with DNase I and precipitated in 70% ethanol before hybridization of 5 × 10^4 cpm/mL of hybridization buffer (50% for-
those from a primary acute myelogenous leukemia sample previously shown to display effective TGF-β binding" (positive control). With membrane proteins isolated from GF-D8 cells; lanes

the manufacturer. Cells were cultured in the presence of DNA-liposomes for 24 hours, washed, and cultured in the presence or absence of SCF with or without TGF-β1 (10 ng/mL each) for an additional 24 hours, followed by analysis of human growth hormone activity (hGH) in cell-free supernatants using an hGH-specific enzyme-linked immunosorbent assay (ELISA; Eurogenetics, Tessenderlo, Belgium).

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA). EMSAs were performed essentially as previously described.21 Brieﬂy, nuclear extracts were prepared from untreated cells and cells exposed to SCF with or without TGF-β1 according to the method detailed by Dignam et al.24 Protein concentrations were determined by the Bradford assay.25 Sense and antisense oligonucleotides (sense, 5’-TTCTAGGTTGAGGATCTCTTCGAGGGAGTCTT-CCATGGTGACGATC-3’; antisense, 5’-GTCACCATGGAGACTCCACCTAGAAGATC-3’; the binding site is underlined) corresponding to position −147 to −118 of the c-jun promoter containing the NF-jun binding site were synthesized, annealed, and end-labeled using the large fragment DNA polymerase (Klenow) in the presence of α[32P]d-CTP. In selected experiments, a double-stranded oligonucleotide containing a mutated NF-jun recognition site (sense, 5’-TTCCTAGGGTTGAGGTCTTTCTGAGGGAGTCTTCCATGGTGACGATC-3’; antisense, 5’-GTCACTAGGTTGAGACTCCACCTAGAAGATC-3’; the mutated basepairs are underlined) was used. The end-labeled oligonucleotides (1 ng; approximately 10,000 cpm) were incubated with 10 μg nuclear proteins in an incubation buffer containing 50 mmol/L HEPES, pH 7.5, 4 mmol/L MgCl2, 1 mmol/L DTT, and 20% (vol/vol) glycerol for 20 minutes at room temperature. In selected experiments, cytoplasmic proteins (20 μg) were used for EMSA. The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel. The gel was dried and exposed to a Kodak X-Omat film at −70°C using an intensifying screen.

Oligonucleotide design, stability assay, and determination of intracellular duplex formation. Antisense experiments were performed essentially as previously described.20 Oligonucleotides corresponding to the translation initiation site of the c-jun gene as well as the respective antisense oligonucleotide and a nonsense oligonucleotide with the same overall basepair composition as the AS oligonucleotide (AS, 5’-TCATAGAAGCTCCAGCCATTCACTTCAC-3’; S, 5’-GTCAGTGACGAGCTTCTATG-3’; NS, 5’-CAAGCTTCAGTCTTAACTCCAGAT-3’) were synthesized, stabilized by

Fig 1. Binding of [3H]-TGF-β1 to membrane proteins of myelogenous leukemia cells detected on a nonreducing PAGE gel. Experiments were performed in the absence (−) or presence (+) of a fivefold excess of “cold” TGF-β1. Lanes 1 and 3 illustrate results obtained with membrane proteins isolated from GF-D8 cells; lanes 2 and 4 those from a primary acute myelogenous leukemia sample previously shown to display effective TGF-β binding28 (positive control).

Fig 2. SCF enhances proliferation of factor-dependent leukemia cells that is inhibited by TGF-β1. GF-D8 cells were serum- and factor-deprived for 18 hours and then exposed to SFSCM with or without SCF (10 ng/mL) in the presence or absence of TGF-β1 (10 ng/mL) for 48 hours with [3H]-thymidine present in the culture medium for the last 6 hours. Cells were harvested and [3H]-thymidine incorporation was quantitated by scintillation counting. Values are expressed as counts per minute (cpm; means ± SD of three independent experiments). Each experiment was performed in triplicate, with SD never exceeding 9%. For further calculation of data, means of triplicates were used. Comparable results were obtained with the Mo7 cell line (data not shown).
TGF-β INHIBITS NF-JUN BINDING

were harvested, incubated with propidium iodide, and analyzed by flow cytometry (FACStar; Becton Dickinson). The percentage of cells being comparable results were obtained with GF-D8 cells (data not shown).

ST2CM uptake of oligonucleotides, oligomers were 5’ end-labeled with tigraphy (HPLC)-purified, and subsequently sequenced.

the addition of thiosulfate groups, high performance liquid chromatography (HPLC)-purified, and subsequently sequenced. To assess uptake of oligonucleotides, oligomers were 5’ end-labeled with γ-ATP [32P] with the bacteriophage T4 polynucleotide kinase and purified by denaturing polyacrylamide gel electrophoresis. A total of 5 × 10^6 cpm of 5’-labeled oligonucleotide was added to 4 × 10^6 cells. Cells were incubated at 37°C in 7% CO₂ in air for up to 24 hours and aliquots were collected at the time points indicated. Cells were pelleted and the supernatant was harvested and saved. Cells were then washed twice in PBS (GIBCO, Grand Island, NY) and pelleted and the cell pellet was lysed in 0.1 mL of Tris-buffered saline (10 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% NaDOSO₄) followed by phenol-extraction. Aliquots of the aqueous phase, the cell wash, and culture medium supernatants were analyzed by liquid scintillation counting. The percentage of oligonucleotides taken up by the cells was calculated by dividing the counts of the aqueous phase by the total of counts (aqueous phase plus cell wash plus culture-medium supernatant). To assess stability of oligonucleotides, aliquots of the aqueous phase were lyophilized and redissolved in 20 μL of loading buffer (80% deionized formamide, 0.01% bromphenol blue, 0.01% xylene cyanol FF) followed by electrophoresis through a 12% denaturing polyacrylamide gel and fluorography.

Experiments designed to analyze intracellular duplex formation were performed as previously described. Briefly, oligomers were 5’ end-labeled as described above to a specific activity of 2 × 10⁷ cpm/µg and added to cells in culture medium at a concentration of 8 µmol/L for 4 hours. After several washing steps in prewarmed PBS, cells were lysed in 100 µL of lysis buffer (10 mmol/L Tris-HCl, pH 7.4; 10 mmol/L NaCl, 3 mmol/L MgCl₂, 0.05% Nonidet P-40, 0.5% SDS, 100 µg proteinase K/mL). As a carrier, 10,000-fold excess of unlabeled oligomer was added. After phenol-chloroform extraction and ethanol precipitation, an SI nuclease protection assay was performed. Products were analyzed on a 15% denaturing polyacrylamide gel. To confirm that duplexes had been formed intracellularly, a control experiment was performed in which an equal amount of cell-associated radioactive activity together with an excess of unlabeled carrier oligomer was added to lysates of cells that had been cultured in the absence of labeled oligonucleotides (“add back control”).

**RESULTS**

GF-D8 cells constitutively display type I and II TGF-β receptors (R), as demonstrated by binding of [125I]-labeled TGF-β1 to membrane proteins (Fig 1). Type I and II TGF-β R were also identified on Mo7 cells (data not shown). GF-D8 and Mo7 cells also exhibit SCF surface binding sites detectable by MoAb YB5.B8 and flow cytometry and prolif-

<table>
<thead>
<tr>
<th>Treatment With</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFSCM + IL-3</td>
<td>79 ± 4</td>
<td>75 ± 5</td>
<td>82 ± 7</td>
<td>28 ± 3</td>
<td>26 ± 2</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>SFSCM</td>
<td>96 ± 4</td>
<td>92 ± 4</td>
<td>96 ± 3</td>
<td>5 ± 1</td>
<td>4 ± 1</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>SFSCM + TGF-β1</td>
<td>91 ± 3</td>
<td>89 ± 5</td>
<td>85 ± 4</td>
<td>7 ± 1</td>
<td>8 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>SFSCM + TGF-β1 + SCF</td>
<td>91 ± 5</td>
<td>90 ± 6</td>
<td>97 ± 3</td>
<td>9 ± 2</td>
<td>10 ± 2</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>SFSCM + SCF</td>
<td>69 ± 3</td>
<td>76 ± 6</td>
<td>83 ± 4</td>
<td>31 ± 3</td>
<td>29 ± 3</td>
<td>17 ± 3</td>
</tr>
</tbody>
</table>

Mo7 cells were cultured in SFSCM with or without SCF (10 ng/mL) and/or TGF-β1 (10 ng/mL) as indicated. After 24, 48, and 72 hours, cells were harvested, incubated with propidium iodide, and analyzed by flow cytometry (FACStar; Becton Dickinson). The percentage of cells being in G2/M, or in S/G2M was calculated. The results represent means of two independent experiments. Standard deviation never exceeded 5%. Comparable results were obtained with GF-D8 cells (data not shown).

**Fig 3.** TGF-β prevents SCF-induced c-jun mRNA accumulation. Factor-dependent leukemia cells were serum- and factor-deprived for 18 hours, followed by exposure to SFSCM with or without SCF (10 ng/mL) in the presence or absence of TGF-β1 (10 ng/mL) for 4 hours. RNA was hybridized with a c-jun-specific cDNA. Hybridization with β-actin controls for the integrity of RNA and comparable RNA loading in single lanes. Shown is a representative blot using GF-D8 cells; comparable results were obtained with the Mo7 cell line (data not shown).

**Fig 4.** TGF-β interferes with the capacity of SCF to transcriptionally enhance the c-jun gene in factor-dependent leukemia cells. GF-D8 cells were serum- and factor-deprived for 18 hours and then exposed to SFSCM with or without SCF (10 ng/mL) in the presence or absence of TGF-β1 (10 ng/mL) for 2 hours. Shown are run-on assays probing nuclei of GF-D8 cells. Nuclei were assayed for transcriptional activity of the c-jun and β-actin gene. To control for unspecific hybridization signals, vector-plasmid was included as negative control (Vector). Comparable results were obtained with Mo7 cells (data not shown).
SCF transactivates the c-jun promoter engaging the NF-jun binding site. (A) Various forms of deleted c-jun promoter constructs, linked to the hGH gene as a reporter gene, were transiently transfected into GF-D8 cells. On transfection, cells were maintained for 24 hours in SFSCM before rhSCF (10 ng/mL) was added in the presence or absence of TGF-β1 (10 ng/mL) for an additional 24 hours. hGH activity was assessed in cell-free culture supernatants by EIA and is expressed as picograms per 10^6 cells per 24 hours after normalization of transfection efficiency with a control plasmid containing the β-galactosidase gene (pSV-BAG). Values are expressed as means ± SD of three independent experiments. (B) GF-D8 cells were transfected with the p-286-c-junhGH promoter construct containing (wt) or not containing (mt) the NF-jun binding site as detailed above. hGH activity was assessed in cell-free supernatants by ELSA and is expressed as picograms per 10^6 cells per 24 hours after normalization of transfection efficiency. Values represent means ± SD of three independent experiments. Comparable results were obtained with Mo7 cells (data not shown).

SCF failed to modulate spontaneous proliferation of serum- and factor-deprived GF-D8 or Mo7 cells on its own when used in concentrations of up to 10 ng/mL, whereas higher concentrations had some toxic effects.

To assess the effect of TGF-β on the proliferative response of factor-dependent cells in more detail, the cell cycle distribution of factor-dependent cells maintained in the presence or absence of SCF, TGF-β, or IL-3 was analyzed over a period of up to 72 hours (Table 1). After serum- and factor-deprivation, 91% of Mo7 cells were shown to arrest in the G0/G1 phase of cell cycle. On addition of SCF, cells were restimulated in that 29% were recruited into S or G2/M phase within 24 hours of SCF stimulation. However, when SCF was combined with TGF-β, 91% to 97% of factor-deprived cells remained in G0/G1 phase of cell cycle, indicating that TGF-β was preventing SCF-mediated cell cycle recruitment. Serum- and factor-deprived cells exposed to TGF-β alone for up to 72 hours also remained in the G0/G1 phase. The viability of factor-dependent cells remained unchanged under all conditions during the 72-hour observation period. When TGF-β was added at 1, 4, 6, 12, or 18 hours after SCF-restimulation, it still prevented SCF-mediated cell cycle recruitment for up to 6 hours, whereas TGF-β added at later time points (12 and 18 hours) failed to inhibit SCF-mediated cell cycle recruitment (data not shown).

TGF-β treatment of GF-D8 and Mo7 cells did not result in any modulation of SCF receptor surface expression (data not shown), suggesting that TGF-β-mediated inhibition of SCF-induced proliferation did not involve transdownregulation of the SCF receptor but occurred most likely through interference with other signal transduction pathways initiated...
TGF-β INHIBITS NF-jun BINDING

Fig 6. The NF-jun binding site confers inducibility to SCF of a heterologous promoter that is inhibited by TGF-β1. A heterologous promoter construct consisting of the herpes thymidine kinase promoter linked to the hGH gene with (pNF-junTKGH) or without the NF-jun binding site inserted 5' of the promoter (pTKGH) were transiently transfected into GF-D8 cells as described above. On exposure of transfectants to SFSCM with or without SCF (10 ng/mL) in the presence or absence of TGF-β1 (10 ng/mL) for 24 hours, reporter gene activity (hGH) was assessed in culture supernatants by ELISA. Results are expressed as picograms per 10⁶ cells per 24 hours after normalization of transfection efficacy. Values represent means ± SD of three independent experiments. Comparable results were obtained with the M07 cell line (data not shown).

These findings suggest that TGF-β was capable of interfering with a signaling pathway initiated by SCF that otherwise leads to transcriptional activation of the c-jun gene. To further clarify the molecular mechanisms leading to SCF-mediated transcriptional activation of the c-jun gene, various forms of deleted promoter constructs were transiently transfected into GF-D8 and M07 cells and reporter gene activity was assessed in transfectants exposed to standard culture medium with or without SCF in the presence or absence of TGF-β (Fig 5A). Pilot experiments, not shown here, had shown that optimum reporter gene activity in transfectants was achieved by exposure to SCF for 24 hours. Transfectants exposed to TGF-β alone or in combination with SCF remained viable during this time period (data not shown). On exposure to SCF, factor-dependent cells responded with transcriptional activation of the c-jun gene that 

by SCF. In this regard it is of note that we have previously shown that the synergistic growth stimulation of AML blasts on exposure to tumor necrosis factor-α (TNF-α) and IL-3 requires functional c-jun/AP-1. However, immunofluorescence analysis, being a semiquantitative technique, may not be sensitive enough to detect TGF-β-mediated downmodulation of SCF-surface binding sites.

To further elucidate the functional role of c-jun/AP-1 in TGF-β-mediated growth inhibition, in a first set of experiments the regulation of the c-jun gene expression by SCF and TGF-β was examined. Northern blot analysis showed that SCF enhanced c-jun mRNA accumulation within 1 hour of exposure. Maximum c-jun mRNA accumulation was observed after 4 to 6 hours, and c-jun mRNA levels declined thereafter (data not shown). In the presence of TGF-β, SCF failed to enhance c-jun mRNA accumulation within 1, 4, or 6 hours (Fig 3 and data not shown). TGF-β did not modulate constitutive c-jun gene expression in GF-D8 and M07 cells on its own during an observation period of 12 hours (data not shown).

Transcriptional run-on assays furthermore indicated that SCF was upregulating the transcriptional activity of the c-jun gene fourfold to fivefold, as determined by densitometric scanning of autoradiographs. The capacity of SCF to increase the transcriptional rate of the c-jun gene was significantly reduced by TGF-β (Fig 4). In line with these findings it was noted that SCF enhanced synthesis of c-jun/AP-1 protein within 8 to 12 hours of exposure. SCF-mediated synthesis of c-jun/AP-1 was inhibited in the presence of TGF-β. Constitutive c-jun/AP-1 protein levels were not modulated by TGF-β alone during a 12-hour observation period (data not shown).

Fig 7. SCF enhances NF-jun binding activity in a dose-dependent fashion. GF-D8 cells were serum- and factor-deprived for 18 hours and then exposed to SFSCM (M) or to medium containing SCF (5 to 100 ng/mL) for 1 hour. Nuclear proteins were prepared and 10 µg nuclear proteins were incubated with a labeled oligonucleotide containing the NF-jun binding site (left panel) or a mutated NF-jun binding site (right panel) as detailed in Materials and Methods.
was predominantly mediated by the region located between position -168 and -128 of the c-jun promoter. Deletion of the c-jun promoter up to position -128 abolished the capacity of SCF to enhance reporter gene activity. When transfectants were exposed to both SCF and TGF-β, SCF failed to enhance transcriptional activity of the c-jun promoter. TGF-β did not modulate the transcriptional activity seen when transfectants had been exposed to standard culture medium only. The region between position -168 and -128 of the c-jun promoter harbors the NF-jun binding site that has previously been identified to regulate c-jun gene transcription in response to TNF-α in acute myelogenous leukemia cells.21

To further substantiate the notion that SCF-mediated transcriptional activation of the c-jun gene engages NF-jun, cells were also transfected with a promoter construct containing a mutated NF-jun binding site (Fig 5B). The capacity of SCF to enhance promoter gene activity in these transfectants was significantly reduced as compared with transfectants carrying the c-jun promoter with an intact NF-jun binding site. The remaining activation on SCF by 1.8-fold is most likely mediated by the AP-1 transcription factor (located at position -72 to -62 within the c-jun promoter), which has previously been shown to confer positive autoregulation of the c-jun gene.26

Moreover, insertion of the NF-jun binding site 5' of the herpes thymidine kinase promoter is sufficient to confer inducibility to SCF of this heterologous promoter construct, whereas the enhancerless construct failed to respond with enhanced reporter gene activity on SCF exposure (Fig 6). Again, exposure of cells to both TGF-β and SCF interfered with the capacity of SCF to enhance promoter gene activity, whereas TGF-β did not modulate constitutive promoter activity on its own.

Gel mobility shift assays showed that SCF enhanced NF-jun binding activity dose-dependently in the range of 5 to 100 ng/mL (Fig 7). Dose-dependency was lost when higher concentrations were used (data not shown). Competition assays using 25-fold molar excess of unlabeled oligonucleotide containing the NF-jun binding site confirmed the specificity of DNA-protein interactions.

The capacity of SCF to enhance NF-jun binding activity was also time-dependent (data not shown). NF-jun binding activity was enhanced within 5 to 10 minutes on exposure to SCF, peaked after 30 to 60 minutes, and returned to starting levels within 4 hours.
TGF-β INHIBITS NF-jun BINDING

When cells had received both SCF and TGF-β, the capacity of SCF to enhance NF-jun binding activity was significantly reduced (Fig 8), whereas TGF-β did not modulate constitutive binding activity on its own. NF-jun has previously been shown to be localized in the cytoplasm from which it translocates to the nucleus on mitogen challenge of the target cell. Similarly, in line with our previous findings using TNF-α as stimulus, we show that SCF induces nuclear translocation of NF-jun in GF-D8 cells as well that was, however, not inhibitable by TGF-β1 (Fig 8).

Taken together, these findings indicate that TGF-β interferes with the capacity of SCF to transcriptionally activate the c-jun gene and to induce accumulation of c-jun mRNA by abolishing SCF-enhanced binding activity of NF-jun. To further characterize the role of c-jun for the proliferative response induced by SCF, the antisense technique was instrumental. Exposure of factor-dependent cells to an antisense (AS) oligodeoxyribonucleotide to c-jun, but not to a sense (S) or nonsense (NS) oligomer leads to intracellular duplex formation followed by efficient and specific inhibition of c-jun/AP-1 synthesis (data not shown), as previously described.26 Factor-dependent cells that had been exposed to SCF along with a c-jun AS oligodeoxyribonucleotide failed to proliferatively respond to SCF (Fig 9). The capacity of the AS oligodeoxyribonucleotide to interfere with the proliferative response was dose-dependent with an optimum inhibition being seen when doses at 10 μmol/L oligomers were used. Higher oligomer concentrations elicited some toxic effects. However, both the sense and the nonsense oligomers failed to prevent SCF-induced proliferation of factor-dependent cells.

DISCUSSION

Confirming our previous results, we show that SCF-induced proliferation of human myelogenous leukemia cells is inhibited by TGF-β.18 In addition, we here show that the capacity of SCF to induce a proliferative response requires the presence of c-jun, as demonstrated by antisense experiments. SCF enhances the transcriptional activity of the c-jun gene and thereby leads to accumulation of c-jun mRNA. Transcriptional activation of c-jun by SCF is predominantly mediated through the NF-jun binding site. Deletion of the NF-jun binding site within the c-jun promoter abolishes the capacity of SCF to activate the c-jun promoter. Moreover, insertion of the NF-jun site 5’ of a heterologous promoter is sufficient to confer inducibility of this promoter to SCF. Activation of both the c-jun promoter as well as the heterologous herpes thymidine kinase promoter by SCF is significantly reduced in the presence of TGF-β. SCF enhances NF-jun binding activity both dose- and time-dependently. The capacity of SCF to enhance NF-jun binding activity is markedly suppressed in the presence of TGF-β.

TGF-β is known to be a growth, differentiation, and morphogenesis factor, depending on the cell type being targeted.1 However, the signaling cascade initiated by TGF-β remains poorly understood. Several surface TGF-β binding proteins have been identified, with type I and II TGF-β receptors being most likely engaged in signal transduction.27-29 The type II receptor harbors a serine/threonine kinase domain for which, so far, no candidate substrates have been identified.30 On exposure to TGF-β, expression of the early response gene c-jun is enhanced, downregulated, or unmodified, depending on the target cell.30-36 Expression of c-jun/AP-1 itself has been linked to proliferation, differentiation, and apoptosis.37-40 However, so far, the requirements of c-jun/AP-1 for these cellular responses to occur have only been conclusively demonstrated with respect to the proliferative response. Transcriptional activation of c-jun accompanies recruitment of resting fibroblasts into the cell cycle, and both c-fos and c-jun are required for G1/G0 transition.41 c-jun has also been proposed to be of importance for progression through late G1 and S phase.42 We have previously shown that the synergistic growth stimulation of fibroblasts by TNF-α and IL-3 relies on the capacity of TNF-α to induce c-jun/AP-1.20 We extend these findings by demonstrating here that expression of c-jun, in addition to that of c-myc and the cdk4 kinase,13,41 is also a molecular target for TGF-β to exert its negative growth regulatory properties. TGF-β-mediated inhibition of SCF-induced cell cycle recruitment appears to occur in mid-G1. Cells that receive TGF-β 8 hours after exposure to SCF no longer respond to TGF-β1, with cell cycle arrest. Taking into account that SCF-mediated accumulation of c-jun mRNA peaks at 4 to 6 hours on exposure of serum- and factor-deprived cells to SCF, these findings suggest that c-jun/AP-1 may play a role in cell cycle recruitment in early to mid G1. The molecular mechanisms by which TGF-β interferes with the capacity of SCF to enhance NF-jun binding activity are currently under more detailed investigation. We presently show that, despite the presence of TGF-β, SCF promotes nuclear translocation of NF-jun,
which is a prerequisite for binding of NF-jun to the c-jun promoter and thereby allows transcriptional activation of the c-jun gene. These findings suggest that TGF-β interferes with the capacity of NF-jun to recognize its binding sequence but does not abolish signaling events leading to its nuclear translocation. One may speculate that SCF-mediated nuclear translocation and DNA binding activity are governed by two distinct signal transduction pathways; one is inhibited by TGF-β, whereas the other one is not. Alternatively, TGF-β may mediate posttranslational modifications of additional nuclear factors that modulate NF-jun binding activity.

REFERENCES

37. Shaw RJ, Doherty DE, Ritter AG, Benedict SH, Clark RA: Adherence-dependent increase in human monocyte PDGF(B) mRNA is associated with increases in c-fos, c-jun, and EGR2 mRNA. J Cell Biol 111:2139, 1990
Transforming growth factor-beta relieves stem cell factor-induced proliferation of myelogenous leukemia cells through inhibition of binding of the transcription factor NF-jun

C Sott, B Dorner, L Karawajew, F Herrmann and MA Brach