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# Regulation of *Socs* Gene Expression by the Proto-oncoprotein GFI-1B

## TWO ROUTES FOR STAT5 TARGET GENE INDUCTION BY ERYTHROPOIETIN*

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**SOCS proteins take part in a classical negative feedback loop to attenuate cytokine signaling. Although STAT family members positively modulate *Socs* gene expression, little else is known about *Socs* gene regulation. Here, we identify functional binding sites for GFI-1B, a proto-oncogenic transcriptional repressor, in the promoters of murine *Socs1* and *Socs3*. Thus, mutating these sites relieved transcriptional repression, as determined by luciferase reporter assays of transiently transfected erythropoietin-responsive 32D-EpoR and HCD57 cells. Furthermore, cotransfection of *Gfi-1B* expression plasmid repressed reporter activity of wild-type (but not mutagenized) *Socs1* and *Socs3* promoters, strongly suggestive of direct GFI-1B binding to these promoters. In addition, overexpression of *Gfi-1B* resulted in reduced transcript levels of *Socs1* and *Socs3*, but not *Socs2* or *Cis*. Upon stimulation with erythropoietin, *Socs* transcripts were rapidly induced, whereas *Gfi-1B* mRNA was downregulated. Interestingly, the latter effect appears to rely on STAT5 activity, but not on phosphoinositide 3-kinase or MAPK pathways. Thus, cytokine-mediated STAT5 activation allows relief of direct repression by GFI-1B of the *Socs1* and *Socs3* promoters, but apparently not of the *Socs2* and *Cis* promoters. This constitutes a previously undescribed mode of controlling cytokine responsiveness, through the direct repression of a tumor suppressor (SOCS1) by a proto-oncoprotein (GFI-1B).**

Responsiveness to cytokines depends upon a balance of positive and negative regulators. The latter includes at least three protein classes. SHP1 (SH2 domain-containing protein-tyrosine phosphatase-1), PIAS (protein inhibitors of activated STAT proteins), and SOCS (suppressors of cytokine signaling) (1). There are at least eight members of the SOCS family, each of which contains a variable N-terminal region, a central SH2 domain, and a conserved C-terminal SOCS box domain (2–8). Several SOCS family members have been shown to negatively regulate cytokine signaling *in vitro* and to result in severe physiological effects when disrupted by gene targeting in mice (9–14).

Although the role of SOCS proteins in modulating cytokine signaling has been extensively studied, less is known about the mechanisms that control *Socs* gene expression. Others have shown that a plethora of cytokines induces *Socs* expression at a transcriptional level and that there are functional binding sites for cytokine-activated STAT (signal transducer and activator of transcription) family members in the promoters of *Cis* (cytokine-inducible SH2 protein) and *Socs3* (15–17). Furthermore, *Socs1* expression is controlled, in part, through translational repression (18, 19). Recent evidence suggests that, in the majority of examined human hepatocellular carcinomas, *Socs1* is transcriptionally silenced through aberrant methylation in its CpG islands (20, 21). Restored *Socs1* expression suppresses both growth rate and anchorage-independent growth of such human hepatocellular carcinoma cells, supporting the notion that SOCS1 may indeed be a tumor suppressor.

We now demonstrate the role of a proto-oncogenic transcriptional repressor, GFI-1B (growth factor independence), in regulating *Socs1* and *Socs3* expression in response to erythropoietin (EPO).[1] *Gfi-1B* was cloned based on its sequence similarity to *Gfi-1*, a target of provirus integration in T-cell lymphoma lines selected for interleukin-2 independence in culture and in primary retrovirus-induced lymphomas (22, 23).

Although similar in their consensus DNA recognition sequences and domain organization, *Gfi-1B* is preferentially expressed in erythropoietic organs such as bone marrow and spleen, whereas *Gfi-1* is more abundant in the lymphopoietic thymus (22). 32D-EpoR and HCD57, two EPO-responsive cell lines, express *Gfi-1B*, but not *Gfi-1*. In response to EPO stimulation, *Socs1*, *Socs2*, *Socs3*, and *Cis* transcript levels increase, whereas *Gfi-1B* levels decline. We provide evidence that GFI-1B directly represses the promoters of *Socs1* and *Socs3* through specific DNA-binding elements and that EPO-induced *Gfi-1B* diminution relies on STAT5 activity, but is independent of phosphoinositide 3-kinase (PI3K) or MAPK pathways. Thus, cytokine-mediated activation of STAT5 allows relief of direct repression by GFI-1B of distinct *Socs* promoters. In summary, we have identified a novel mechanism for controlling responsiveness to cytokines; a mechanism for differential regulation of *Socs* genes; and new targets to account for the oncogenic properties of GFI-1 family members, which have previously been shown to directly repress genes that control the cell cycle and apoptosis (22, 24).

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[1] The abbreviations used are: EPO, erythropoietin; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; EpoR, erythropoietin receptor; EMSA, electrophoretic mobility shift assay.

## EXPERIMENTAL PROCEDURES

*Plasmids and Mutagenesis*—Site-directed mutagenesis was achieved using the QuikChange™ XL site-directed mutagenesis kit (Stratagene) following the manufacturer's instructions. *Gfi-1B* cDNA was derived from wild-type C57BL/6 mouse spleen RNA using the oligonucleotides 5′-ATGAGGATCCCCAGGGCAAGTGTGGAGGTTCGTGG-3′ and 5′-CTGAGAATTCAGCGAGACACACTAAAGCAGGCGGC-3′ and the Advantage™ One-Step RT-PCR kit (CLONTECH). After sequence confirmation and digestion with *Bam*HI and *Eco*RI, it was cloned into the mammalian expression plasmid pMex(neo) (provided by Dr. Stephanie Watowich) to generate pMex-*Gfi-1B* or into pcDNA3.1 (Invitrogen). pMex-P2A-*Gfi-1B* was derived from pMex-*Gfi-1B* using the primer 5′-GCACGCAGAAAAATGGCACGGTCCTTTCTAGTG-3′ and its complementary primer.

Genomic DNA clones containing *Socs1* or *Socs2* were isolated from a 129(J1) genomic library (25), and the 5′-flanking regions were mapped. To clone the *Socs1* 5′-flanking region into a luciferase reporter plasmid, a 3.1-kb fragment obtained by *Hin*dIII digestion and blunting, followed by partial *Xho*I digestion, was inserted into *Sma*I-*Xho*I pGL3-basic (Promega) to generate Clone 9. The *Socs2* 5′-flanking region was cloned by inserting a 6.5-kb *Bgl*II-*Bst*XI (blunted) fragment into pGL3-basic.

For mutagenesis of the *Socs1* promoter, the following oligonucleotides and their respective complementary oligonucleotides were employed: 5′-AGGACAAACTGGGTCACGAAACCACACAGT-3′ for the upstream GFI-1B-binding site, 5′-ACAGCTTTTTTTGGGGTCACAGC CCCGCCCGGGGCC-3′ for the downstream GFI-1B-binding site, 5′-CTAGCTAGCCGGGTACGAAGAAGGGTCGAGATTGC-3′ for the upstream STAT-binding site, and 5′-GAGAGAACCCGAAAGACTAGGC-GGAAAGAGAAACCG-3′ for the downstream STAT-binding site. For each construct, a 1.9-kb *Sac*II-*Esp*I fragment containing the appropriate mutation(s) was subcloned into the wild-type backbone and subsequently sequenced to ensure that no unexpected mutations were present.

For mutagenesis of the *Socs3* promoter, the primer 5′-GCTCTACT-GGGTCACAGGTCATGACTAGTCCTTGCTCATGG-3′ and its complementary primer were employed to mutate the GFI-1B-binding site in the previously described Clone 6, containing 2.8 kb of the *Socs3* 5′-flanking region in the pGL3-basic vector (17). A 1.5-kb *Afl*II-*Bst*XI fragment harboring this mutation was subcloned into the backbone of either wild-type Clone 6 or Clone 6D2, in which a critical STAT-binding element is destroyed (17).

*Cell Culture and Stable Cell Line Generation*—The culture conditions for 32D cells that ectopically express either wild-type or mutant erythropoietin receptor (EpoR), and HCD57 cells, which naturally express EpoR, have been described (26, 27). NIH3T3 cells were passaged in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To generate cell lines that constitutively overexpress *Gfi-1B*, pMex-*Gfi-1B* (or the pMex vector as a control) was used to electroporate 32D-EpoR or HCD57 cells (25 $\mu$g of DNA/$10^7$ cells; pulsed at 25 microfarads and 400 V). Stable transfectants were selected in 0.6 mg/ml G418 (for 32D-EpoR cells) or 1.0 mg/ml G418 (for HCD57 cells). To generate cell lines that inducibly overexpress *Gfi-1B*, a *Kpn*I-*Eco*RV *Gfi-1B* fragment was shuttled from pcDNA3.1 into the metallothionein promoter-containing MT-CB6$^+$(neo) eukaryotic expression vector (supplied by Dr. Frank Rauscher III). After electroporation, G418 selection, limiting dilution, and expansion, single clones were tested for their -fold induction of the *Gfi-1B* transcript upon addition of 100 $\mu$M zinc sulfate. To generate cell lines that stably express dominant-negative Stat5, the C-terminally truncated ΔStat5B (provided by Dr. Alice Mui) was subcloned into pcDNA3.1. The resulting construct was linearized with *Pvu*I and used to electroporate 32D-EpoR or HCD57 cells as described above. Stable transfectants were selected in G418 as described above and analyzed as subpools rather than single clones.

*Transient Transfections and Reporter Assays*—32D-EpoR, HCD57, or NIH3T3 cells were transfected with 5 $\mu$g of total plasmid DNA/$10^6$ cells using Effectene™ transfection reagent (QIAGEN Inc.) following the manufacturer's guidelines. For mutant promoter analyses, 4.5 $\mu$g of carefully quantitated pGL3-basic-based plasmid, which encodes firefly luciferase, was cotransfected with 0.5 $\mu$g of pRL-TK (Promega), which encodes *Renilla* luciferase and is used as an internal control reporter for normalizing transfection efficiency. For transient *Gfi-1B* overexpression studies, 4 $\mu$g of either pMex-*Gfi-1B* or pMex-P2A-*Gfi-1B* was cotransfected with 0.5 $\mu$g of *Socs* promoter-containing pGL3-basic and 0.5 $\mu$g of pRL-TK. Cell lysates were prepared 36 h post-transfection and analyzed for luciferase activity using the Dual-Luciferase® reporter assay system (Promega). The indicated relative reporter activities take into account normalized transfection efficiency and, where applicable, discrepancies in plasmid size.

*Northern Blot Analysis*—10 $\mu$g of total RNA, isolated from cells using Trizol reagent (Invitrogen), was separated by gel electrophoresis; transferred to nylon membranes; and hybridized with [$\alpha$-$^{32}$P]dCTP-labeled DNA probes, derived from full-length cDNA template using the Prime-It® II random primer labeling kit (Stratagene). After hybridization for 2 h at 65 °C using QuikHyb® hybridization solution (Stratagene), blots were washed twice for 20 min at 65 °C with 0.1× SSC and 0.1% SDS.

*Electrophoretic Mobility Shift Assay (EMSA)*—Nuclear extracts were prepared from HCD57 or 32D-EpoR cells as described (26). *Gfi-1B* was *in vitro* transcribed and translated by the TNT® T7 coupled reticulocyte lysate system (Promega). EMSAs were done with the following oligonucleotides, each annealed to its complementary strand: 5′-AGGACAAACTGAATCACGAAACCACACAGT-3′ for the upstream GFI-1B-binding site in the *Socs1* promoter; 5′-ACAGCTTTTTTTGGA-ATCACAGCCCCGCCCGGGGCC-3′ for the downstream GFI-1B-binding site in the *Socs1* promoter; 5′-GCTCTACTGGGTCACAAATCATG-ACTAGTCCTTGCTCATGG-3′ for the *Socs3* promoter GFI-1B-binding site, 5′-GCTAGCCGGGTTCCAAGAAGGGTCGAGAT-3′ for the upstream STAT-binding site in the *Socs1* promoter, and 5′-CCCGAAAG-ACTTGCCGGAAAGAGAAACCG-3′ for the downstream STAT-binding site in the *Socs1* promoter. Mutant oligonucleotides were used for competition studies: AATC to GGTC for GFI-1B binding and TTNC-NNNAA to TANGNNNAA for STAT5 binding. Wild-type double-stranded oligonucleotides were end-labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (New England Biolabs Inc.). Either 10 $\mu$g of nuclear extract or one-tenth of the *Gfi-1B* translated *in vitro* from RNA derived from 1 $\mu$g of pcDNA3.1-*Gfi-1B* was incubated in binding buffer (10 mM HEPES, pH 7.6, 0.5 mM dithiothreitol, 50 mM NaCl, 0.05% (v/v) Nonidet P-40, 10% (v/v) glycerol, 50 ng/$\mu$l poly(dI-dC) (Amersham Biosciences, Inc.), and 2.5 mg/ml nonfat milk) at room temperature for 20 min prior to addition of labeled probe. For supershift studies, nuclear extracts were preincubated on ice for 1 h with 1 $\mu$g of anti-STAT5 or anti-GFI-1B antibody (Santa Cruz Biotechnology). To compete for protein binding, excess wild-type or mutant double-stranded oligonucleotides were included in binding buffer (40–200-fold relative to labeled probe). Labeled probe (25,000 cpm) was added, and the binding reaction was incubated at room temperature for 30 min. Samples were then electrophoresed at 160 V on nondenaturing 5% polyacrylamide gels at 4 °C for 3 h in 0.5× Tris borate/EDTA, followed by gel drying and autoradiography.

## RESULTS

*Transcriptional Repression of Select Socs Promoters by GFI-1B*—Our initial goal was to dissect the mechanisms of *Socs1* transcriptional induction by EPO. During the initial stages of this analysis, smaller promoter fragments were found to possess greater activity than larger fragments (data not shown), suggesting that sequences upstream of the proximal promoter may possess binding sites for functionally relevant repressors. With MatInspector Version 2.2 (28), we found two potential GFI-1/GFI-1B-binding sites, each containing a 100% conserved AATC core and overall 8 out of 12 nucleotide matches to the consensus sequence determined by binding of random oligonucleotides to a glutathione *S*-transferase-tagged GFI-1 or GFI-1B fusion protein (22, 29). As shown in Fig. 1*A*, these two sites reside at −900 to −911 and at −763 at −774 relative to the transcriptional start site previously determined (19), whereas two putative STAT-binding elements reside at −103 to −111 and at −467 to −475 (30). *Gfi-1B* (but not *Gfi-1*) is highly expressed in two EPO-responsive cell lines, 32D-EpoR and HCD57 (26, 27), as determined by Northern analysis (Fig. 1*B*). This concurs with previous findings that *Gfi-1B* is preferentially expressed over *Gfi-1* in mouse erythropoietic organs, bone marrow and spleen (22). From then on, we focused our interests on *Gfi-1B*.

The aforementioned cell lines, which are EPO-dependent for proliferation and survival and die within 24 h of EPO deprivation, were deprived of EPO (but no other components of their regular growth medium) for 16 h, followed by stimulation with 1 unit/ml EPO (Fig. 1*C*). Whereas *Socs* transcripts were induced within 1 h, *Gfi-1B* mRNA was rapidly down-regulated (the mRNA half-life is <1 h in the absence or presence of EPO

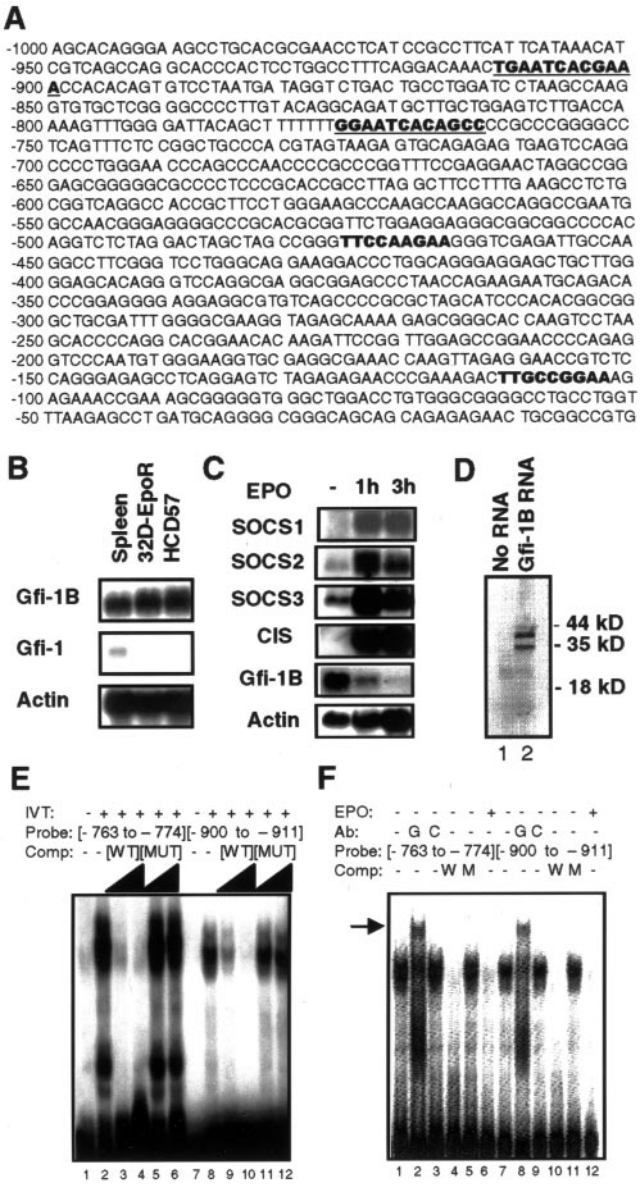


FIG. 1. **GFI-1B-binding sites in the murine *Socs1* promoter and regulation of *Gfi-1B* and *Socs* transcripts by EPO.** *A*, the mouse *Socs1* promoter sequence, which corresponds to bp 10873–11872 of GenBank™/EBI Data Bank accession number Z47352, contains two putative GFI-1B-binding sites (22), which are in *boldface* and *underlined*, and two putative STAT-binding elements (TTNCNNNAA) (30), which are in *boldface*. *B*, *Gfi-1B* (but not *Gfi-1*) is expressed in 32D-EpoR and HCD57 cells, as determined by Northern analysis described under "Experimental Procedures." *C*, 32D-EpoR cells were deprived of EPO for 16 h, followed by stimulation with 1 unit/ml EPO and RNA isolation at the indicated time points for Northern analysis. *D*, [$^{35}$S]methionine-labeled, *in vitro* translated GFI-1B was electrophoresed by 10% SDS-PAGE and visualized by autoradiography. *Lane 1* shows the results of a reaction without added *Gfi-1B* expression plasmid; *lane 2* contains the *in vitro* translation products of pcDNA3.1-*Gfi-1B*. *E*, the following $^{32}$P-labeled double-stranded oligonucleotide probes were used in EMSA: a 36-bp probe including the putative GFI-1B-binding site spanning −763 to −774 of the *Socs1* promoter (*lanes 1–6*) and a 30-bp probe including the putative GFI-1B-binding site spanning −900 to −911 (*lanes 7–12*). *Lanes 1* and *7* contain the oligonucleotide probe and reticulocyte lysate only (negative control). In *lanes 3*, *5*, *9*, and *11*, the corresponding oligonucleotide competitors were added at 40-fold excess, whereas in *lanes 4*, *6*, *10*, and *12*, they were added at 200-fold excess. *IVT*, *in vitro* translation product; *Comp*, oligonucleotide competitor; *WT*, wild-type unlabeled oligonucleotide competitor; *MUT*, mutant (AATC to GGTC) unlabeled oligonucleotide competitor. *F*, the same probes described for *E* were tested for binding to nuclear extracts. Each lane used 10 $\mu$g of nuclear extract from 32D-EpoR cells deprived of EPO for 16 h (*lanes 1–5* and *7–11*) or deprived of EPO and then stimulated with 1 unit/ml EPO for 2 h (*lanes 6* and *12*). For supershift analyses, nuclear extracts were incubated with either anti-GFI-1B antibody (*G*; *lanes 2* and *8*) or an irrelevant control antibody (*C*; *lanes 3* and *9*). In *lanes 4*, *5*, *10*, and *11*, the corresponding oligonucleotide competitors were added at 100-fold excess. *Ab*, antibody; *Comp*, competitor; *W*, wild-type competitor; *M*, mutant competitor.

as determined by actinomycin D treatment) (data not shown). The inverse correlation between *Socs* and *Gfi-1B* transcript levels upon EPO stimulation is consistent with the latter repressing the former.

To assess the functional relevance of these potential GFI-1B-binding sites, we performed EMSAs using *in vitro* translated GFI-1B. Fig. 1*D* (*lane 2*) shows two major [$^{35}$S]methionine-labeled products, the larger one corresponding to the expected full-length GFI-1B protein and the shorter one, as seen previously (22), probably arising from an internal AUG start codon and thus expected to contain the C-terminal DNA-binding domain. A 36- or 30-bp probe corresponding to *Socs1* promoter fragments containing the downstream (−763 to −774) (Fig. 1*E*, *lanes 1–6*) or upstream (−900 to −911) (*lanes 7–12*) GFI-1B-binding site, respectively, was used for binding either *in vitro* translated GFI-1B (Fig. 1*E*, +) or reticulocyte lysate negative control (−) in the absence or presence of a 40- or 200-fold excess of unlabeled wild-type or mutant (AATC to GGTC) competitor. The two major shifted bands, likely corresponding to the two *in vitro* translation products, diminished when the binding reaction was co-incubated with excess wild-type (but not mutant) competitor. Thus, we established specific binding of GFI-1B to the appropriate promoter elements *in vitro*.

These conclusions were further supported when nuclear extracts of 32D-EpoR or HCD57 cells were employed (Fig. 1*F*). Cells were deprived of EPO as described above to enhance accumulation of endogenous GFI-1B, and nuclear extracts were prepared as described previously (26). To establish the identity and specificity of GFI-1B binding, equal amounts of nuclear extract were co-incubated either with anti-GFI-1B antibody (Fig. 1*F*, *G*) or irrelevant control antibody (*C*) or with 100-fold excess unlabeled wild-type (*W*) or mutant (*M*) competitor. Anti-GFI-1B (but not control) antibody could supershift the EMSA complexes (Fig. 1*F*, *lanes 2* and *8*, *arrow*), confirming GFI-1B binding. This complex was diminished upon specific competition (Fig. 1*F*, *lanes 4* and *10*). Furthermore, GFI-1B binding was indiscernible with nuclear extracts prepared from EPO-stimulated cells (Fig. 1*F*, *lanes 6* and *12*). Thus, down-regulation of *Gfi-1B* transcript levels (Fig. 1*C*) parallels down-regulation of protein activity, strengthening the biological relevance of our findings.

To determine the functionality of these sites and their interplay with STAT-binding elements in a cellular setting, we compared the activities of various *Socs1* promoter-driven luciferase reporter constructs, which were generated as described under "Experimental Procedures." First of all, STAT5 binding to the putative elements identified in Fig. 1*A* was confirmed by EMSA using nuclear extracts from EPO-stimulated cells (Fig. 2*A*), in analogy to the experiment described in Fig. 1*F*. Either element could indeed bind to STAT5, as confirmed by supershift with anti-STAT5 antibody (*S*; Fig. 2*A*, *lanes 2* and *7*, *arrow*), but not with an irrelevant control antibody (*C*; *lanes 3* and *8*), and by specific competition (*lanes 4*, *5*, *9*, and *10*).

The parental construct (Clone 9) contains 2.4 kb upstream of the *Socs1* transcriptional start site and untranslated exon 1, but ends immediately upstream of the start codon. This reporter activity is strictly EPO-dependent (data not shown), and all values in Fig. 2*B* derive from reporter assays of 32D-EpoR cells continuously passaged in EPO and therefore represent steady-state EPO signaling. Mutating either one or both of the

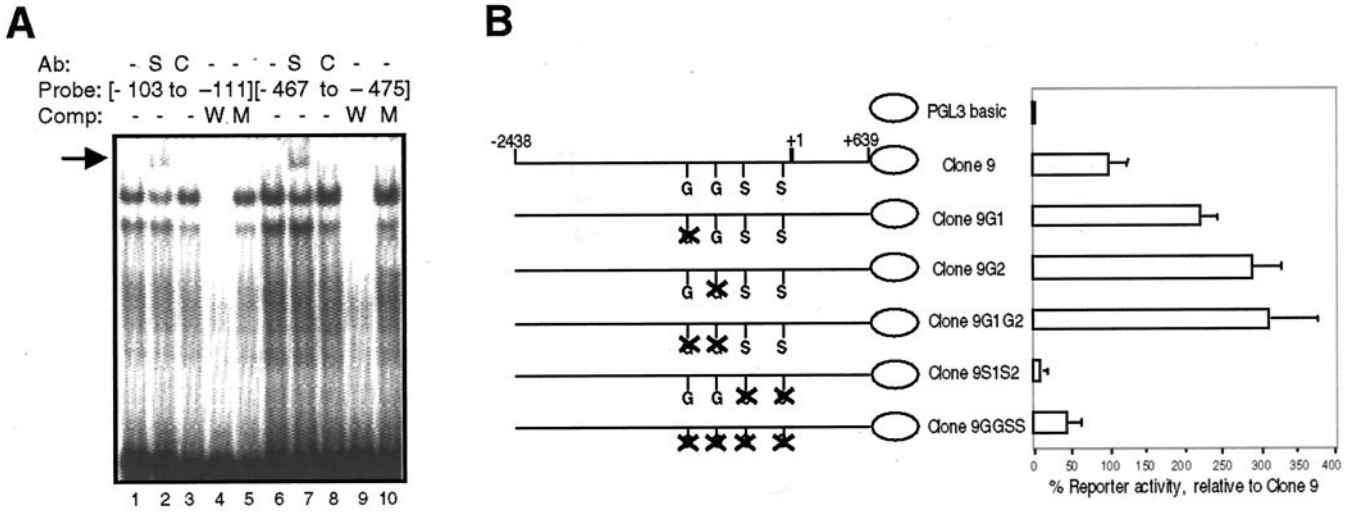


FIG. 2. **Confirmation of STAT5 binding to putative elements and reporter activities of various constructs containing the 5′-region of murine *Socs1*.** *A*, in each lane, 10 $\mu$g of nuclear extract from EPO-stimulated cells was tested for binding to 29-bp probes containing putative STAT5-binding sites spanning either −103 to −111 or −467 to −475 of the *Socs1* promoter. For supershift studies, nuclear extracts were incubated with either anti-STAT5 antibody (*S*; *lanes 2* and *7*) or an irrelevant control antibody (*C*; *lanes 3* and *8*). In *lanes 4*, *5*, *9*, and *10*, the corresponding oligonucleotide competitors were added at 100-fold excess. *Ab*, antibody; *Comp*, competitor; *W*, wild-type competitor; *M*, mutant (TTNCNNNAA to TANGNNNAA) competitor. *B*, 32D-EpoR cells continuously passaged in 1 unit/ml EPO were transiently cotransfected with the indicated plasmid and a *Renilla* luciferase-encoding plasmid for normalization. Values are based on the results of eight independent experiments and are presented as the means ± S.D. Similar results were observed with HCD57 cells (data not shown). *G*, GFI-1B-binding site; *S*, STAT-binding site. *Ovals* represent the luciferase reporter.

GFI-1B-binding sites (AATC to GGTC) resulted in a 2.2–3.1-fold enhancement of reporter activity, suggesting relief of transcriptional repression and cooperativity between the two GFI-1B-binding sites (Clones 9G1, 9G2, and 9G1G2) (Fig. 2*B*). Furthermore, mutating both STAT5-binding elements (TTNCNNNAA to TANGNNNAA) reduced reporter activity to ~11% of the wild type (Clone 9S1S2 *versus* Clone 9). Interestingly, mutating all four elements resulted in reporter activity that was 45% of the wild type, a 4.0-fold increase over the STAT5-binding element mutant (Clone 9GGSS compared with Clone 9S1S2). This suggests that *Socs1* promoter activity can be significant even in the absence of STAT5 binding and that GFI-1B plays a critical role in setting the base-line level of wild-type promoter activity. Furthermore, the ability of GFI-1B to repress reporter activity in the absence of STAT5 binding suggests that its mechanism of action does not solely, if at all, rely on prevention of STAT5 binding. Similar results were obtained with HCD57 cells (data not shown).

To determine whether overexpression of *Gfi-1B* alone is sufficient to repress *Socs1* promoter activity and to assess its role in the regulation of other *Socs* promoters, Clone 9 (Fig. 3*A*, *bars 4–6*), a *Socs2* promoter reporter (*bars 7–9*), or a *Socs3* promoter reporter (*bars 10–12*) was cotransfected with the empty vector pMex (*bars 1*, *4*, *7*, and *10*), pMex-*Gfi-1B* (*bars 2*, *5*, *8*, and *11*), or pMex-P2A-*Gfi-1B* (*bars 3*, *6*, *9*, and *12*), the latter encoding a debilitating point mutation in the SNAG (*Snail*/*Gfi-1*) repressor domain (22, 31). To normalize transfection efficiency, pRL-TK, which lacks consensus binding sites for GFI-1B, was used. Overexpressing *Gfi-1B* (but not the P2A mutant) repressed *Socs1* and *Socs3* (but apparently not *Socs2*) promoter activity, suggesting that GFI-1B differentially regulates *Socs* genes and requires a functional repressor domain to do so. Indeed, examination of 2.0 kb of the 5′-flanking sequence of *Socs2* revealed no obviously potential GFI-1B-binding sites. Similar results were seen with both 32D-EpoR and HCD57 cells.

Because gene expression can be regulated by distal or downstream as well as proximal or upstream elements, we attempted to confirm the above conclusions by surveying endogenous *Socs* transcript levels in the absence or presence of *Gfi-1B* overexpression. Fig. 3*B* shows the effects of sustained *Gfi-1B* overexpression on the induction of *Socs* transcripts after withdrawal of (−) and subsequent stimulation with EPO. *Socs1* and *Socs3* transcript levels were slightly reduced at all time points, whereas *Socs2* and *Cis* were unaltered upon comparison of subpools of 32D-EpoR cells stably overexpressing *Gfi-1B* (Fig. 3*B*, *right panels*) with subpools harboring the empty vector (*left panels*). Furthermore, we generated multiple 32D-EpoR lines that stably express *Gfi-1B* from an inducible metallothionein promoter. Continuously passaged in EPO, these cells were treated with 100 $\mu$M zinc sulfate to induce *Gfi-1B* overexpression, which again resulted in decreased *Socs1* and *Socs3* transcript levels, but unaltered *Socs2* and *Cis* transcript levels (Fig. 3*C*).

This led us to analyze the *Socs3* promoter, which has been cloned and characterized (17). Sequence analysis revealed the presence of one putative GFI-1B-binding site located at −2057 to −2068 relative to the transcriptional start site (Fig. 4*A*). Indeed, mutation of the AATC core to GGTC resulted in a 1.8-fold enhancement of reporter activity (Clone 6-G compared with wild-type Clone 6) in 32D-EpoR and HCD57 cells continuously passaged in EPO. Furthermore, Clone 6D2, in which both putative STAT-binding elements are disrupted, resulted in ~37% promoter activity relative to Clone 6. Again, mutating the GFI-1B-binding site resulted in an ~2-fold increase in promoter activity in the STAT binding-defective mutant (Clone 6D2-G *versus* Clone 6D2). Thus, the repressive activity of GFI-1B does not appear to rely on STAT binding to the *Socs3* promoter. To confirm the binding specificity of the relevant promoter fragment for *in vitro* translated GFI-1B (Fig. 1*D*) or GFI-1B from nuclear extracts, EMSA was conducted using a 41-bp fragment of the *Socs3* 5′-flanking region, which includes the putative GFI-1B-binding element. Supershift studies as well as specific and nonspecific competitors were employed, analogous to the experiments described for Fig. 1*E* (Fig. 4*B*) and Fig. 1*F* (Fig. 4*C*). Again, GFI-1B specifically bound to the relevant promoter fragment *in vitro*.

To assess the directness of GFI-1B regulation of the *Socs1* and *Socs3* promoters, highly transfectable NIH3T3 fibroblasts were also used. These cells express *Socs1* and *Socs3*, but not *Socs2*, *Cis*, or *Gfi-1B*, as determined by Northern analysis (data

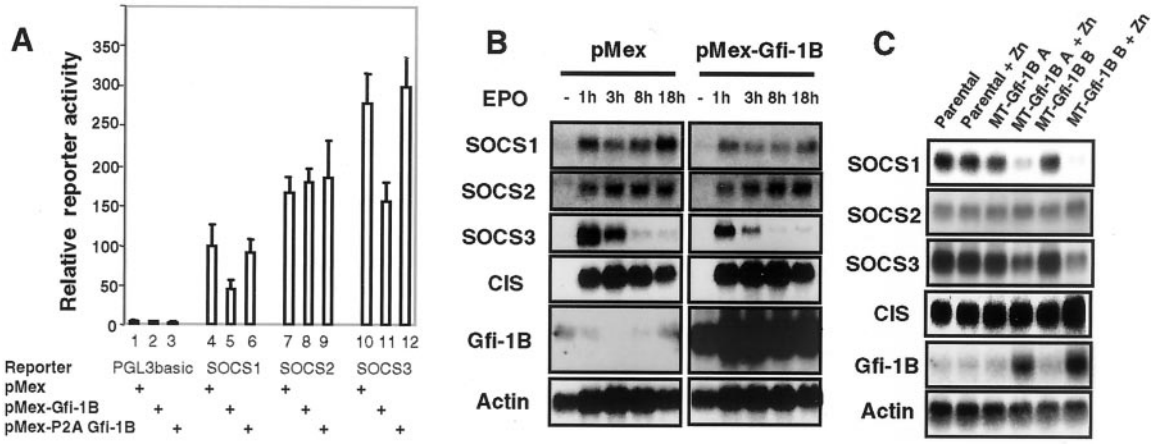


FIG. 3. **Differential expression of *Socs* genes in response to *Gfi-1B* overexpression.** *A*, 32D-EpoR cells were transiently cotransfected with 1) pRL-TK for normalization; 2) the pMex, pMex-*Gfi-1B*, or pMex-P2A-*Gfi-1B* expression plasmid (the latter encodes a mutant form of *Gfi-1B* defective in transcriptional repression); and 3) a pGL3-basic-based reporter construct containing either *Socs1* (Clone 9), *Socs2*, or *Socs3* promoter. Normalized relative reporter activity values (means ± S.D.) are based on the results of five independent experiments and were similar in 32D-EpoR and HCD57 cells. *B*, 32D-EpoR cells stably harboring pMex (*left panels*) or pMex-*Gfi-1B* (*right panels*) were deprived of EPO for 16 h and then stimulated with 1 unit/ml EPO for the indicated times, at which point, RNA was isolated for Northern analysis. *C*, parental 32D-EpoR cells or individual Clones A and B, which inducibly overexpress *Gfi-1B* from a metallothionein promoter, were grown in 1 unit/ml EPO in the absence or presence of 100 $\mu$M zinc sulfate for 24 h. RNA was isolated and subjected to Northern blot analysis.

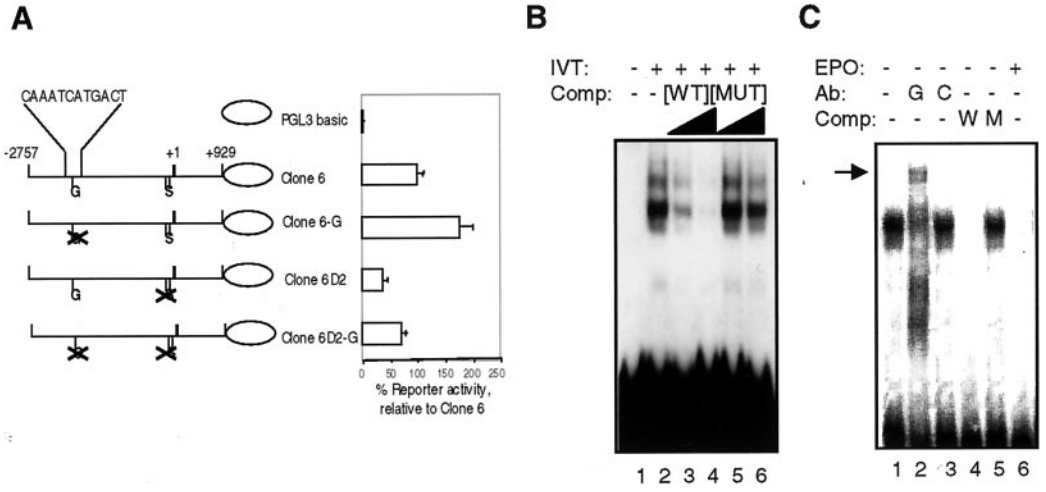


FIG. 4. **Role of GFI-1B in regulating *Socs3* promoter activity.** *A*, a putative GFI-1B-binding site spans −2057 to −2068 of the murine *Socs3* promoter, relative to the transcriptional start site previously determined (17). *G*, GFI-1B-binding site; *S*, STAT-binding site. *Ovals* represent the luciferase reporter. Clone 6 consists of the wild-type full-length *Socs3* 5′-flanking region previously characterized, whereas Clone 6D2 was derived by deleting two putative STAT-binding elements in Clone 6 (17). The core sequence AATC was mutated to GGTC in Clones 6-G and 6D2-G, which were derived from Clones 6 and 6D2, respectively. Transient transfections and luciferase assays were performed as described for Fig. 2*B*. Values are based on the results of six independent experiments (means ± S.D.) and were similar in 32D-EpoR and HCD57 cells. *B*, a 41-bp probe containing this putative GFI-1B-binding site was used for EMSA as described in the legend to Fig. 1*E*. *Lane 1* contains the oligonucleotide probe and reticulocyte lysate only (negative control). In *lanes 3* and *5*, the corresponding oligonucleotide competitors were added at 40-fold excess and, in *lanes 4* and *6*, at 200-fold excess. *IVT*, *in vitro* translation product; *Comp*, oligonucleotide competitor; *WT*, wild-type unlabeled oligonucleotide competitor; *MUT*, mutant (AATC to GGTC) unlabeled oligonucleotide competitor. *C*, this probe was used for EMSA with nuclear extracts as described in the legend to Fig. 1*F*. Each lane used 10 $\mu$g of nuclear extract from 32D-EpoR cells deprived of EPO for 16 h (*lanes 1–5*) or deprived of EPO and then stimulated with 1 unit/ml EPO for 2 h (*lane 6*). For supershift analysis, nuclear extracts were incubated with either anti-GFI-1B antibody (*G*; *lane 2*) or an irrelevant control antibody (*C*; *lane 3*). In *lanes 4* and *5*, the corresponding oligonucleotide competitors were added at 100-fold excess. *Ab*, antibody; *Comp*, competitor; *W*, wild-type competitor; *M*, mutant competitor.

not shown). As shown in Fig. 5, mutant promoters lacking functional GFI-1B-binding sites were not derepressed compared with their respective wild-type promoters, presumably because of the lack of endogenous *Gfi-1B* expression (*bars 3* and *5* for *Socs1* and *bars 7* and *9* for *Socs3*). However, cotransfection of pMex-*Gfi-1B* repressed the promoter activity of both *Socs1* (Fig. 5, *bars 3* and *4*) and *Socs3* (*bars 7* and *8*). On the other hand, promoters in which the GFI-1B-binding sites have been mutated failed to respond to exogenous *Gfi-1B* (*bars 5* and *6* for *Socs1* and *bars 9* and *10* for *Socs3*), confirming direct binding of GFI-1B to both the *Socs1* and *Socs3* promoters.

Consistent with GFI-1B-mediated repression of select *Socs* promoters, SOCS proteins and GFI-1B manifested opposing biological effects. Whereas stable overexpression of *Gfi-1B* in 32D-EpoR cells resulted in enhanced proliferation (Fig. 6), overexpression of *Socs1* or *Socs3* reduced cell proliferation.[2] Although the former effect can be explained, in part, by previous observations that GFI-1B represses the $p21^{Waf1}$ promoter (22), it also is compatible with our findings that GFI-1B represses the promoters of certain *Socs* family members, which negatively regulate cytokine-induced cell proliferation.

*STAT5-dependent Diminution of Gfi-1B Levels in Response to EPO*—Having shown that *Gfi-1B* levels rapidly declined in

[2] A. G. Jegalian and H. Wu, manuscript in preparation.

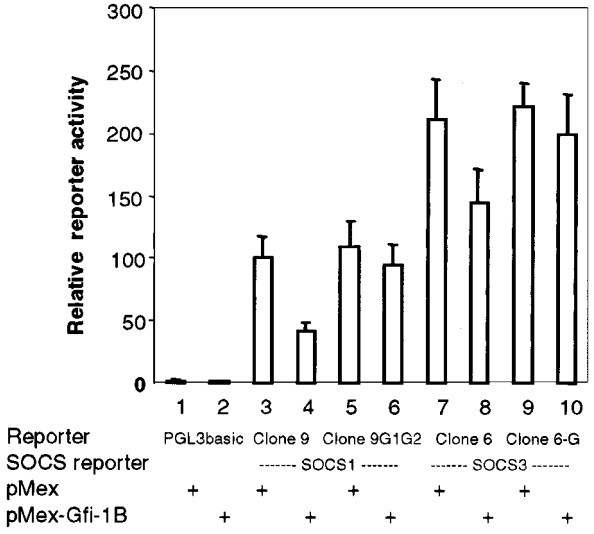


FIG. 5. **Direct repression of *Socs1* and *Socs3* promoters by GFI-1B.** NIH3T3 cells were cotransfected with 1) either pMex or pMex-*Gfi-1B* and 2) wild-type *Socs1* (Clone 9) or *Socs3* (Clone 6) promoter reporter plasmid or with versions of these reporters in which the putative GFI-1B-binding sites are mutated (Clones 9G1G2 and 6-G, respectively). Luciferase activities were measured as described under "Experimental Procedures." Values are based on four independent experiments.

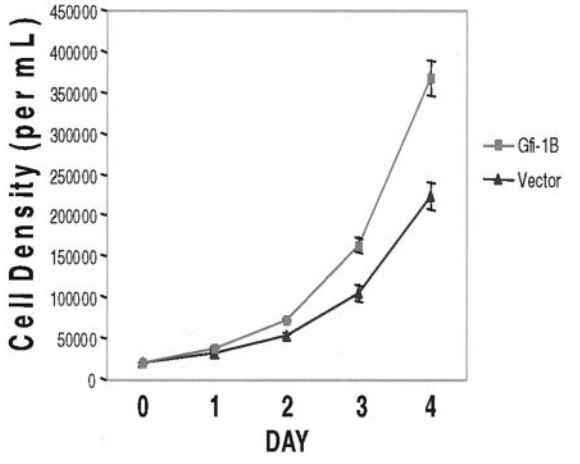


FIG. 6. **Enhanced cell proliferation upon *Gfi-1B* overexpression.** On Day 0, 32D-EpoR cells stably overexpressing *Gfi-1B* or harboring just the vector were plated at 20,000 cells/ml in medium containing 0.1 unit/ml EPO. Live cells (excluding trypan blue) were counted on each day, and the results are based on five different subpools/sample.

response to EPO (Fig. 1*C*), we dissected the pathways downstream of EPO that might account for this effect. The first approach made use of 32D cell lines that express similar levels of previously characterized EpoR mutants, each of which carries a single cytoplasmic tyrosine residue (26, 32). Either $Tyr^{343}$ or $Tyr^{401}$ sufficiently mediates near-maximal activation of STAT5, but fails to activate MAPK or PI3K, whereas $Tyr^{464}$ and $Tyr^{479}$ fail to activate STAT5, but are sufficient for activation of MAPK and PI3K (32, 33). As shown in Fig. 7*A*, wild-type EpoR (but not the F8 form, in which all eight cytoplasmic tyrosines were converted to phenylalanines) allowed *Gfi-1B* down-regulation upon EPO stimulation. Thus, EPO-induced *Gfi-1B* reduction requires these tyrosine residues, which are phosphorylated in response to EPO stimulation. $Y^{343}F7$ or $Y^{401}F7$, like wild-type EpoR, allowed EPO-induced *Gfi-1B* reduction, whereas $Y^{464}F7$ or $Y^{479}F7$ failed to do so. Thus, there is a tight correlation between STAT5 activation and *Gfi-1B* decline.

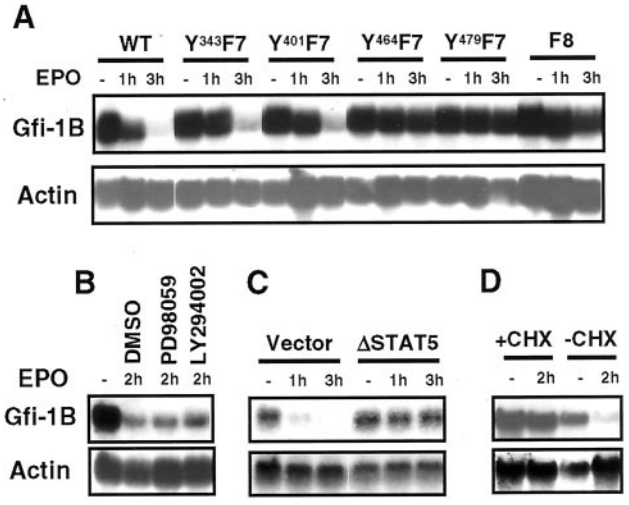


FIG. 7. **Requirement of STAT5 for down-regulation of *Gfi-1B* in response to EPO stimulation.** *A*, 32D cells expressing similar levels of the indicated EpoR mutants, which have been described previously (26), were deprived of EPO for 16 h and then stimulated with 1 unit/ml EPO. Northern analysis was performed as described under "Experimental Procedures." *WT*, wild type. *B*, after 2 h of pretreatment and in the continued presence of $Me_2SO$ carrier control (*DMSO*), 5 $\mu$M LY294002 (PI3K inhibitor), or 50 $\mu$M PD98059 (MAPK inhibitor), EPO-starved 32D-EpoR cells were stimulated with 1 unit/ml EPO. RNA was isolated after 2 h and subjected to Northern analysis. *C*, 32D-EpoR cells stably carrying the pcDNA3.1 empty vector or stably expressing C-terminally truncated dominant-negative *Stat5* (Δ*Stat5*) (34) were deprived of EPO for 16 h and then stimulated with 1 unit/ml EPO for either 1 or 3 h. Northern analysis was performed as described under "Experimental Procedures." *D*, 32D-EpoR cells were deprived of EPO for 14 h and then pretreated or not with 50 $\mu$g/ml cycloheximide (*CHX*) for 30 min before stimulation with 1 unit/ml EPO for 2 h. Northern analysis was performed as described under "Experimental Procedures."

The second approach made use of chemical inhibitors of MAPK (PD98059) and PI3K (LY294002) and a dominant-negative form of STAT5. 32D-EpoR cells were cultured without EPO for 14 h and then pretreated for 2 h with $Me_2SO$ carrier control, 5 $\mu$M LY294002, or 50 $\mu$M PD98059, followed by 2 h of treatment with 1 unit/ml EPO in the continued presence of chemical inhibitors. Northern analysis revealed that neither chemical inhibitor prevented EPO-mediated *Gfi-1B* down-regulation; and thus, this effect appears to be independent of PI3K and MAPK (Fig. 7*B*). To assess the role of STAT5 in *Gfi-1B* regulation, 32D-EpoR cells stably carrying the pcDNA3.1 vector or stably expressing C-terminally truncated dominant-negative *Stat5* (Δ*Stat5) (34) were deprived of EPO for 16 h and then stimulated with EPO for either 1 or 3 h. As shown in Fig. 7C*, whereas *Gfi-1B* down-regulation occurred in the vector-transfected controls, it did not occur in cells overexpressing Δ*Stat5*. Taking together the results of Fig. 7 (*A–C*), we conclude that the C terminus of STAT5 is necessary for EPO-mediated *Gfi-1B* reduction, whereas MAPK and PI3K are neither sufficient nor necessary for *Gfi-1B* diminution.

To determine whether STAT5-dependent *Gfi-1B* down-regulation requires new protein synthesis, 32D-EpoR cells were deprived of EPO for 14 h and then pretreated with 50 $\mu$g/ml cycloheximide before EPO stimulation for 2 h. As shown in Fig. 7*D*, cycloheximide prevented EPO-mediated *Gfi-1B* down-regulation. Similar results were seen with HCD57 cells. Thus, it is likely that STAT5 protein, whose half-life is several hours (data not shown), activates intermediate target genes rather than acting directly to suppress *Gfi-1B* levels.

## DISCUSSION

SOCS family members are critical regulators of cytokine signaling, and recent evidence supports the notion of at least

one member, SOCS1, being a tumor suppressor (20, 21). *Gfi-1* and *Gfi-1B*, on the other hand, are proto-oncogenes, the former being implicated in T-cell lymphomagenesis and the latter found to be a target of provirus integration in a subset of B-cell lymphomas induced by Moloney murine leukemia virus in E$\mu$-*myc* transgenic, *pim-1*/*pim-2* null mice (22, 35). In this study, we have demonstrated a link between *Gfi-1B* and *Socs1* as well as *Socs3*. Namely, we have identified functional binding sites for GFI-1B in the promoters of *Socs1* (−763 to −774 and −900 to −911) and *Socs3* (−2057 to −2068). Furthermore, disrupting the GFI-1B-binding sites in STAT5 binding-defective promoter mutants still allows relief of the repressive effects of GFI-1B, suggesting that GFI-1B does not act solely, if at all, by interfering with STAT5 binding to its corresponding sites.

Indeed, the mechanism of action of GFI-1B as a transcriptional repressor is not clear. GFI-1B, like GFI-1, contains a 20-amino acid N-terminal SNAG repressor domain, and it has been demonstrated for GFI-1 (but not for GFI-1B) that its repressive function may be position- and orientation-independent (31). Recently, GFI-1 was shown to interact with PIAS3 (which specifically inhibits STAT3-mediated transcription of target genes), to inhibit PIAS3 action, and therefore to enhance STAT3-mediated transcriptional activation (36). Extending this finding to GFI-1B, however, is not necessarily warranted because the SNAG domain of GFI-1 is not sufficient for binding to PIAS3, and the sequence similarity between GFI-1 and GFI-1B is limited to the SNAG domain and C-terminal zinc fingers (22).

Our finding that GFI-1B is an important negative regulator of the *Socs1* promoter may be compatible with recent evidence suggesting that transcriptional silencing of *Socs1* through aberrant methylation in its CpG islands may contribute to oncogenesis (20, 21). It would be interesting to determine, for instance, if GFI-1B interacts with one of the components of the methyl-CpG-binding MeCP1 complex to stabilize it (37). One must search for GFI-1B-binding proteins to begin to address such issues. However, given the relatively long distances of GFI-1B-binding sites from the transcriptional start sites of target genes (1.5 kb for p21$^{Waf1}$, 0.8–0.9 kb for *Socs1*, and 2.1 kb for *Socs3*), association with a complex silencer region is likely in each case.

In addition, we have shown that EPO down-regulates *Gfi-1B* transcript levels through a mechanism independent of PI3K and MAPK, but requiring the STAT5 C terminus, which contains its transcriptional activation domain. Indeed, new protein synthesis is required for STAT5-mediated *Gfi-1B* down-regulation, which is maximal at 1–2 h of EPO stimulation.

Combining our findings (along with those of others) reveals a scheme whereby STAT5 can control cytokine-response genes through two mechanisms. Although our studies are confined to EPO signaling, we speculate that such a scheme is more general, given the multitude of cytokines that activate STAT5. Furthermore, others have demonstrated that stimulation of myelomonocytic M1 cells with interleukin-6, which activates STAT3, results in *Gfi-1B* down-regulation (22). As shown in Fig. 8, in the absence of cytokine signaling (indicated by *dashed lines*), GFI-1B is abundant and represses the promoters of STAT5 targets such as *Socs1*, *Socs3*, and p21$^{Waf1}$ (22, 38). Upon cytokine stimulation (*solid lines*), activation of STAT5, in addition to causing direct activation of its target genes, allows *Gfi-1B* levels to decline, thereby relieving direct repression of these promoters. It would be interesting to determine whether other transcriptional repressors regulate those STAT target genes that are not regulated by GFI-1B. STAT5 phosphorylation is maximal within minutes of cytokine stimulation,

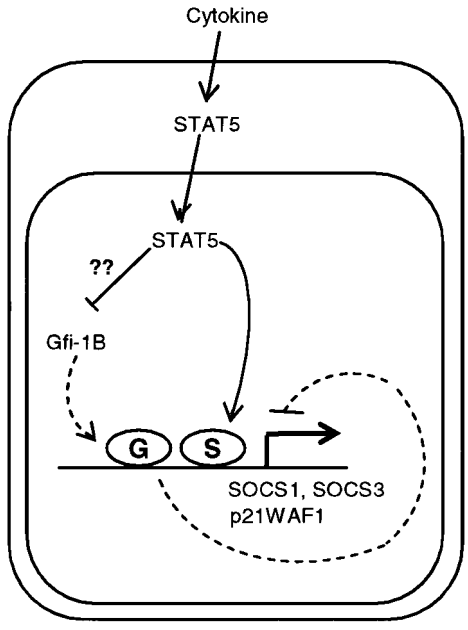


FIG. 8. **Contribution of GFI-1B to cytokine responsiveness.** *G*, GFI-1B protein; *S*, STAT5 protein. *Dashed lines* refer to events predominant in the absence of cytokine signaling, and *solid lines* refer to events favored by cytokine signaling. *Gfi-1B* is expressed in myeloid cells, including 32D and HCD57. In the absence of EPO stimulation, high *Gfi-1B* levels are responsible for the repression of the promoters of several STAT target genes, including p21$^{Waf1}$, *Socs1*, and *Socs3*. Upon EPO addition, STAT5 is activated, resulting in, through new protein synthesis (*??*), reduced *Gfi-1B* levels and therefore release of its inhibitory effects on STAT target genes. Thus, STAT5 up-regulates the transcript levels of target genes through at least two mechanisms, one through direct binding and activation and one through down-regulation of transcriptional repressors such as GFI-1B.

whereas *Gfi-1B* down-regulation is maximal after 1–2 h of cytokine stimulation. Thus, initial induction of *Socs* genes is attributable to transcriptional activation of their promoters by STAT5, whereas maintenance of *Socs1* and *Socs3* levels can be achieved, in part, through relief of transcriptional repression by GFI-1B. These results underscored the importance of multi-level control of *Socs* gene expression in tightly regulated cytokine-mediated effects.

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