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Differential Roles of SOCS Family Members in EpoR Signal Transduction

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ABSTRACT

To elucidate the roles of suppressor of cytokine signaling (SOCS) family members in erythropoietin (EPO) signaling, we explored SOCS gene regulation, mRNA stability, and protein function in two EPO-responsive hematopoietic cell lines. Using two independent approaches, one involving inhibition of specific signaling molecules and the other employing cell lines that express particular EpoR mutants and thereby activate only subsets of signaling cascades, we demonstrate that induction of SOCS1, SOCS2, SOCS3, and cytokine-inducible SH2-containing protein (CIS) in response to EPO stimulation appears to depend on Stat5 but not on mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K). SOCS4 expression, in contrast, does not appear to be EPO inducible. Furthermore, we show differential stabilities of SOCS transcripts, with SOCS2 the longest-lived and SOCS1 and CIS the least stable, and provide evidence in support of EPO-independent expression of SOCS3 and SOCS4. In order to understand the effects of SOCS on EPO-mediated effects, we generated multiple stable cell lines that inducibly express particular SOCS proteins. Overexpression of SOCS1, SOCS3, or CIS negatively regulates EPO-mediated cell proliferation, Stat5 phosphorylation, and activation of a Stat-dependent luciferase reporter. In contrast, SOCS2 is less effective, and SOCS4 is ineffective at counteracting EPO-mediated events. Thus, we have demonstrated differential regulation and function of various SOCS family members in EPO-dependent hematopoietic cells.

INTRODUCTION

Suppressor of cytokine signaling (SOCS) family members are induced in response to a broad range of cytokines and, in turn, may diminish cytokine signaling. Thus, they partake in a classic negative feedback loop to control the duration and intensity of cytokine responses and are involved in cross-talk between different cytokine systems.[1–3] There are at least eight members of this family (cytokine-inducible SH2-containing protein [CIS] and SOCS1–7), each of which contains a variable N-terminal region, a central Src homology 2 (SH2) domain, and a conserved C-terminal SOCS box domain.[4] The current study systematically examines the differential regulation of SOCS gene expression in response to erythropoietin (EPO) stimulation and the roles of different SOCS family members in modulating EPO function.

EPO is a glycoprotein hormone vital for the differentiation of committed erythroid progenitor cells. It signals through the EPO receptor (EpoR), a member of the cytokine receptor superfamily, which lcaks catalytic activity. EpoR association with Janus kinase 2 (Jak2) permits receptor phosphorylation on ligand stimulation and activation of downstream events. These signaling cascades include those mediated by phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 5 (Stat5) (for review, see ref. 5). On phosphorylation, Stat5, a latent transcription factor, dimerizes and translocates into the nucleus, where it activates transcription of target genes, including the founding member of the SOCS family, CIS.

Early reports have documented the induction of SOCS1, SOCS2, SOCS3, and CIS transcripts in mouse bone marrow cells treated with EPO,[6] and CIS provides a model for understanding SOCS gene regulation. The proximal promoter of CIS contains four Stat5-binding sites, all of which are necessary for EPO-inducible activation.[7–10] Additionally, a dominant negative mutant of Stat3 reduces the induction of SOCS1 mRNA in response to interleukin-6 (IL-6) or leukemia inhibitory factor (LIF) in M1 cells.[11] Finally, a Stat1/Stat3-binding element, located −72 to −64 of the SOCS3 promoter, was shown to be essential for LIF stimulation of SOCS3 production.[12] SOCS1 gene expression is negatively controlled by translational repression[13,14] and, through transcriptional repression, by the

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proto-oncoprotein Gfi-1B.[15] However, the roles, if any, of the MAPK and PI3K pathways in SOCS gene regulation are unclear.

Disruption by gene targeting of several SOCS family members causes severe physiologic effects.[16–23] Based on these studies, SOCS3 appears to be the family member most relevant to EPO signaling, as according to one report, SOCS3 null mice exhibit severe erythrocytosis, whereas SOCS3-transgenic mice are anemic.[20] One group showed, using Ba/F3 lymphoid cell lines that ectopically express various EpoR mutants, that SOCS3 binds directly to Jak2 as well as to Y401 of the activated EpoR and inhibits EPO-dependent cell proliferation and Stat5 activation.[24]

SOCS1-deficient, SOCS2-deficient, and CIS-deficient mice, on the other hand, do not exhibit obvious erythroid defects. This is despite findings that CIS tightly binds to phosphorylated Y401 of the EpoR and negatively regulates EPO signaling when overexpressed in cell lines.[7,25,26] Thus, complete dissection of the contribution of SOCS proteins to signaling requires either generation of animals that lack multiple SOCS family members or a biochemical approach, which is essential if clinical therapy with SOCS-like molecules for disturbances in cytokine responsiveness is to be explored.

The current study addresses two important issues: (1) the mechanisms of SOCS gene induction in response to EPO stimulation and (2) the involvement of multiple SOCS family members in EPO/EpoR signaling. Using two independent EPO-responsive hematopoietic cell lines, we have dissected the signaling cascades contributing to SOCS induction in response to EPO and have demonstrated differential stabilities of SOCS transcripts and different degrees of inhibition of EpoR signaling by overexpression of distinct family members.

MATERIALS AND METHODS

Plasmids

FLAG epitope-tagged mouse SOCS1, SOCS2, SOCS3, SOCS4, or CIS cDNA, each in the mammalian expression plasmid pEF, was generously provided by Dr. Doug Hilton. Subcloning into the metallothionein promoter-containing eukaryotic expression vector pMT-CB6+(neo) (provided by Dr. Frank Rauscher, III) was achieved using XbaI sites, and the resulting clones were verified for correct orientation.

Cell culture and proliferation assays

32D cells that have been engineered to express either wild-type or mutant murine EpoR and HCD57 cells, which naturally express the EpoR, have been described.[27,28] 32D-derived cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 5% WEHI-3B-conditioned medium (as a source of IL-3). HCD57 cells were grown in Iscove's modified Dulbecco's medium (IMDM) containing 20% FBS and 1 U/ml recombinant human erythropoietin (rHuEPO) (Amgen, Thousand Oaks, CA). For EPO stimulation assays, cells were first washed three times in RPMI/10% FBS (for 32D cells) or in IMDM/20% FBS (for HCD57 cells) to remove traces of IL-3 or EPO. After incubation in cytokine-free medium at 37°C and 5% CO_2 for 14–16 h, cells were stimulated with 1 U/ml rHuEPO and counted at given times. Cells were counted using either a hemacytometer, after staining with trypan blue to assess viability, or using a Coulter Z1 Particle Counter (settings at 9–12 μm to exclude dying cells and clumps) (Coulter Electronics, Hialeah, FL). To inhibit PI3K and MAPK pathways, cells were pretreated with DMSO vehicle control, 20 μM LY294002 (PI3K inhibitor), or 50 μM PD98059 (MAPK inhibitor) for 30 min at 37°C before EPO addition.

Generation of stable cell lines

For generating cell lines that inducibly overexpress various SOCS family members, pMT-SOCS1, pMT-SOCS2, pMT-SOCS3, pMT-SOCS4, or pMT-CIS was linearized with PvuI and used to electroporate 32D-EpoR cells (25 μg DNA for 10^7 cells, pulsed at 25 μF and 400 V). Stable transfectants were selected in 0.6 mg/ml G418, and after limiting dilution and expansion, single clones were tested for their fold induction of the relevant SOCS transcript on addition of 100 μM 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 pcDNA 3.1. The resulting construct was linearized with PvuI and used to electroporate 32D-EpoR or HCD57 cells, as described. Stable transfectants were selected in G418 as described (1.0 mg/ml for HCD57 cells) and analyzed as subpools rather than single clones.

Transient transfections and reporter assays

32D-EpoR or HCD57 cells were transfected with 5 μg total plasmid DNA per 10^6 cells, using Effectene™ Transfection Reagent (Qiagen, Chatsworth, CA) and following the manufacturer's guidelines. For transient SOCS overexpression studies, 4 μg of either pEF vector or pEF-SOCS expression plasmid was cotransfected with 0.5 μg SOCS1 promoter-containing PGL3 basic[15] and 0.5 μg pRL-TK (Promega, Madison, WI) to normalize transfection efficiency. Cell lysates were prepared 36 h posttransfection and analyzed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega).

Northern blot analysis

Total RNA (10 μg), isolated from cells using Trizol Reagent (Life Technologies, Gaithersburg, MD), was separated by gel electrophoresis, transferred to nylon membranes, and hybridized with α^{32}P-dCTP-labeled DNA probes, derived from full-length cDNA template using the Prime-It® II Random Primer Labeling Kit (Stratagene, La Jolla, CA). After hybridizing for 2 h at 65°C using QuikHyb® Hybridization Solution (Stratagene), blots were washed twice for 20 min at 65°C using 0.1× SSC/0.1% SDS. The relative abundance of transcripts was determined using a PhosphorImager (ImageQuant™ software, Molecular Dynamics, Sunnyvale, CA) and standardized against actin.

Western blot analysis

After being washed once with phosphate-buffered saline (PBS), cells were lysed on ice in a buffer composed of 20 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After incubating for 30 min on ice, lysates were cleared of debris by centrifugation at 15,000*g* for 30 min. Care-

fully quantitated protein lysates (50 μg per sample) were boiled for 5 min in SDS-PAGE sample buffer and then resolved by SDS-PAGE. This was followed by protein transfer to a nitrocellulose membrane, which was blocked with 5% nonfat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at ambient temperature. The blot was then incubated with antiphospho-Stat5 antibody (Cell Signaling), following the manufacturer's guidelines. After several washes, a 1:3000 dilution of antirabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA) was added and incubated for 1 h at ambient temperature. This was followed by several washes, enhanced chemiluminescence (ECL) (Cell Signaling Technology, Beverly, MA), and exposure to film. The blot was stripped with a buffer composed of 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 min, washed, blocked with 5% nonfat milk in TBS-T, and then reprobed with anti-Stat5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1 μg/ml in 5% nonfat milk/TBS-T overnight at ambient temperature. ECL was performed as described, and after stripping the blot, it was reprobed with anti-FLAG M2 monoclonal antibody (mAb) (Sigma, St. Louis, MO). ECL followed incubation with 1:5000 antimouse HRP-conjugated secondary antibody (Bio-Rad).

RESULTS

A survey of SOCS expression

In order to acquire an initial sense of the relevance of SOCS to EpoR signaling, we surveyed the expression of five of eight known SOCS family members in two EPO-dependent hematopoietic cell lines, HCD57 and 32D-EpoR. As shown in Figure 1, all five members are highly expressed in 32D-EpoR cells. SOCS1, SOCS2, SOCS3, and CIS ar EPO inducible, whereas SOCS4 is not. To further study the regulation of SOCS gene expression, we used pharmacologic inhibitors of signaling pathways known to be controlled by EpoR activation. As shown in Figure 1A, neither PD98059 (MAPK pathway inhibitor) nor LY294002 (PI3K pathway inhibitor) reduces SOCS transcript

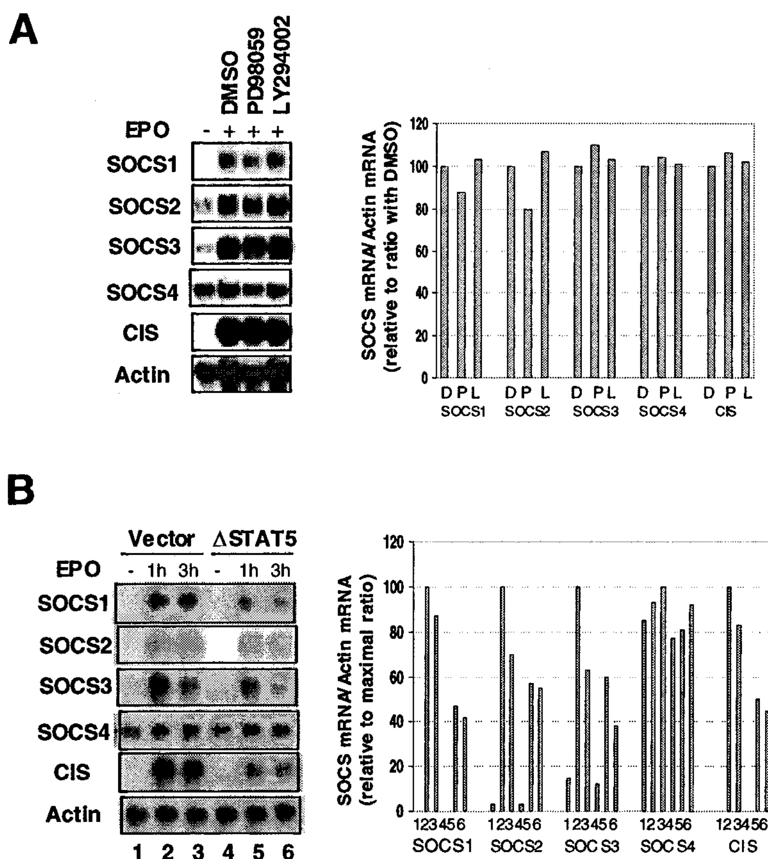

FIG. 1. Effects of specific signaling pathway inhibitors or dominant negative Stat5 on SOCS transcript induction. (**A**) 32D-EpoR cells were washed three times to remove cytokine, then cultured in the absence of EPO for 8 h, at which point cells were pretreated with either PD98059 (50 μM), LY294002 (20 μM), or DMSO carrier control for 30 min at 37°C, followed by the addition of EPO to 1 U/ml for 2 h, in the continued presence of either DMSO alone or chemical inhibitors. Northern analysis was conducted as described. Quantitative values were obtained by PhosphorImage analysis and are normalized against actin. D, DMSO; P, PD98059 (MAPK pathway inhibitor); L, LY294002 (PI3K pathway inhibitor). (**B**) Three subpools of 32D-EpoR cells stably harboring either pcDNA 3.1 vector or a C-terminally truncated dominant negative version of Stat5 (ΔSTAT5) were deprived of cytokine for 15 h, followed by stimulation with 1 U/ml EPO for 1 or 3 h, at which point RNA was isolated. Northern analysis was conducted, and normalized values for SOCS signal intensities for each lane are reported at right. The data shown are representative of three independent experiments.

induction (relative to actin), even at maximal recommended doses that significantly inhibit phosphorylation of AKT and ERK, two directly downstream signaling molecules of the PI3K and MPAK pathways, respectively.[15,29–31] Also downstream of EpoR is Stat5, which is known to activate SOCS3 and CIS transcription. Through the generation of cell lines that overexpress ΔSTAT5, a dominant negative version of Stat5 lacking the C-terminal transactivation domain, we studied the roles of Stat5 in controlling other members of the SOCS family. On EPO stimulation, only half-maximal induction of SOCS1, SOCS2, SOCS3, and CIS was observed in cells that overexpress ΔSTAT5 (Fig. 1B). These results suggest that SOCS induction occurs through Stat5 activation but does not rely on PI3K or MAPK signaling pathways. Furthermore, although SOCS4 is expressed in these cell lines, it does not appear to be a Stat5-dependent or EPO-inducible gene.

Dissection of signaling cascades involved in SOCS induction

Because pharmacologic inhibitors may have nonspecific effects, we took advantage of cell lines that express equivalent amounts of various EpoR mutants.[27] These lines were derived from the IL-3-dependent 32D murine myeloid progenitor cell line, which normally does not express EpoR but can proliferate in response to EPO on ectopic expression of wild-type (WT) EpoR. The mutants included F8, in which all eight cytoplasmic tyrosines (Y) are converted to phenylalanines (F), as well as single tyrosine-containing EpoR variants (Y^xF7). Y343 or Y401 is required for maximal Stat5 activation but fails to activate MAPK or PI3K, whereas Y464 and Y479 are not sufficient to activate Stat5 but are essential for activation of MAPK and PI3K.[32,33] As shown in Figure 2, WT EpoR but not the F8 form allows upregulation of SOCS1, SOCS2, SOCS3, and CIS on EPO stimulation. Thus, EPO-dependent SOCS induction requires the presence of EpoR cytoplasmic tyrosine residues, which are phosphorylated in response to EPO stimulation. It should be noted that F8 as well as $Y^{464}F7$ and $Y^{479}F7$ allow some Stat5 activation (8% of that with WT), which likely accounts for basal levels of SOCS induction.[32] EpoR mutant $Y^{343}F7$ or $Y^{401}F7$, either of which supports near-maximal levels of Stat5 activation, allows maximal SOCS induction, whereas mutant $Y^{464}F7$ or $Y^{479}F7$ fails to do so. Thus, consistent with the results from the studies with pharmacologic inhibitors and dominant negative Stat5 (Fig. 1), this genetic approach further validates the role of Stat5 activation in SOCS induction.

EPO dependence and relative stabilities of SOCS transcripts

Unlike SOCS1 and CIS, SOCS2 and SOCS3 transcripts are detectable in the absence of EPO stimulation or maximal Stat5 activation (Fig. 2), which may reflect cytokine-independent expression or expression in response to unidentified factors in serum or greater transcript stability than seen with SOCS1 or CIS. Thus, the relative stabilities of SOCS transcripts were examined by treating cells with actinomycin D, an inhibitor of mRNA synthesis, for various times, in the absence or presence of EPO. Figure 3 shows the results with HCD57 cells, although similar trends were obtained with 32D-EpoR. On removal of EPO stimulation and in the absence of actinomycin D (Fig. 3, lanes 1–5), SOCS1 and CIS transcripts disappear within 1–3 h, whereas SOCS2, SOCS3, and SOCS4 transcripts are maintained substantially for at least 8 h. The stabilities of SOCS transcripts apparently do not depend on EPO, as similar profiles are seen for each family member on actinomycin D treatment regardless of the absence or presence of EPO (Fig. 3, lanes 6–10 vs. lanes 11–15). One is inclined to conclude that SOCS3 and SOCS4 transcription must be induced, at least to a certain extent, in the absence of EPO, possibly in response to other serum factor(s). The basal level of SOCS2, on the other hand, is due to its prolonged half-life.

Inhibition of EPO-mediated events by SOCS

We next addressed the roles that SOCS proteins may play in the regulation of EpoR signaling. The pilot experiment entailed

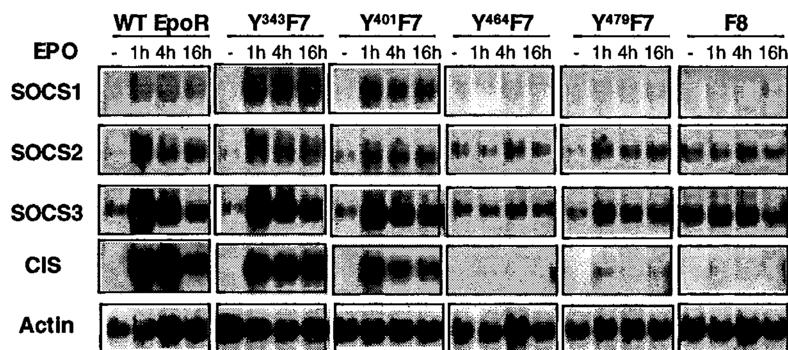

FIG. 2. Time course expression of SOCS genes in response to EPO in cell lines that express previously characterized EpoR mutants. 32D cells that express similar levels of previously characterized EpoR mutants[27,32,33] were maintained in IL-3, then deprived of cytokines for 15 h, followed by stimulation with 1 U/ml rHuEPO for the indicated times. RNA was isolated at these points and subjected to Northern analysis using probes derived from full-length cDNA. IL-3 stimulation caused a similar pattern of induction for all cell lines and was similar to EPO-induced SOCS expression in 32D-WT EpoR (not shown), indicating that the effects seen are EPO mediated. Activation of particular signaling pathways, as described in the text, has been attributed to each of the EpoR variants.[32,33]

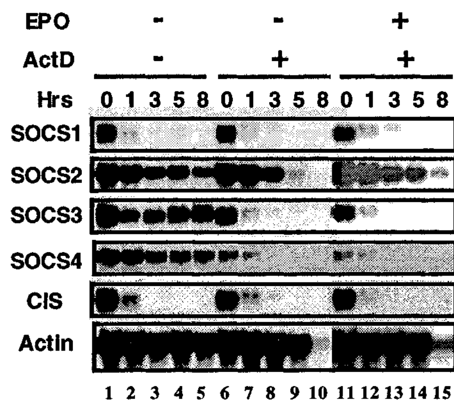

FIG. 3. Relative stabilities and EPO dependence of SOCS transcripts. HCD57 cells were either washed three times with cytokine-free medium and cultured in the absence of EPO for the indicated times (lanes 1–5) or cultured without EPO but with 10 μg/ml actinomycin D for the indicated times (lanes 6–10) or cultured continuously in the presence of 1 U/ml EPO but with actinomycin D added for the indicated times (lanes 11–15). RNA was isolated and subjected to Northern analysis.

transient cotransfection of 32D-EpoR cells with (1) SOCS expression plasmid, (2) pRL-TK plasmid for normalizing transfection efficiency, and (3) a luciferase reporter plasmid of SOCS1 promoter activity, which has been shown to be EPO dependent and to contain functional Stat-binding sites.[15] As shown in Figure 4A, SOCS1, SOCS3, and CIS significantly downmodulate reporter activity ($p < 0.005$), whereas SOCS2 is less efficient ($p < 0.05$), and SOCS4 is ineffective. Similar results were seen with HCD57 cells. Because myeloid cells are in general difficult to transiently transfect at efficiencies adequate for biochemical and cellular analyses, we generated stable cell lines that overexpress various SOCS family members from a zinc-inducible promoter.

Thus, each SOCS cDNA was subcloned into the pMT-CB6+(neo) vector, and the resulting pMT-SOCS1, pMT-SOCS2, pMT-SOCS3, pMT-SOCS4, and pMT-CIS were each introduced into 32D-EpoR cells. Single clones were screened by Northern analysis for their fold induction of the appropriate SOCS transcript in the presence of 100 μM zinc sulfate (data not shown). For each construct, two independent clones (A and B) were tested for EPO-mediated effects: (1) EPO-dependent cell proliferation and (2) Stat5 phosphorylation.

Figure 4B presents the results of proliferation assays of these clones on addition of 30 μM zinc. Cell numbers were counted daily for 3 days to measure cell proliferation. As no significant cell death was observed in all samples during the assay period (i.e., <1% based on trypan blue staining), decelerated increase in cell number likely reflects reduced cell proliferation rather than enhanced cell death. All clones grow at a similar rate in the absence of zinc (data not shown). However, overexpression of SOCS1, SOCS3, or CIS, on zinc addition, reduces the rate of cell proliferation. SOCS2 overexpression reduces cell proliferation but not to as great an extent as other family members, whereas SOCS4 overexpression does not significantly alter cell proliferation.

The fact that all the overexpressed proteins should be FLAG epitope tagged allows direct comparisons of overexpressed levels of various SOCS proteins. Cells were continuously passaged in EPO in the absence or presence of 30 μM zinc sulfate for 24 h, followed by protein extraction and Western analysis. As shown in Figure 4C (top), zinc induces expression of FLAG-containing proteins of appropriate molecular weights. Endogenous SOCS protein levels, however, are very low and undetectable by Western analysis unless concentrated by immunoprecipitation (data not shown). Thus, the amount of SOCS protein produced is comparable among clones. The same blot was probed with both phospho-Stat5 and Stat5 antibodies. Zinc at 30 μM is not cytotoxic, and Stat5 levels are not changed with zinc treatment per se or on SOCS overexpression (Fig. 4C, bottom). However, in certain cell lines that inducibly overexpress SOCS, particularly those that overexpress SOCS1, SOCS3, and CIS, Stat5 phosphorylation is strongly reduced or not even detectable on zinc treatment, with consistent results between the two clones per construct (Fig. 4C, middle). On the other hand, Stat5 phosphorylation is not substantially altered on SOCS2 or SOCS4 overexpression. Thus, the specific effects of different SOCS on EPO-induced Stat5 phosphorylation are consistent with their activities in the EPO-dependent reporter gene expression assay (Fig. 4A) and with their effects on EPO-dependent cell proliferation (Fig. 4B).

DISCUSSION

Cytokine signaling is tightly regulated, with multiple levels of both positive and negative control. The latter is executed by at least three protein classes, namely, the SH2-containing protein tyrosine phosphatase 1 (SHP1), the protein inhibitors of activated Stats (PIAS), and the SOCS. In this study, we have shown that although at least five SOCS family members are transcribed in two EPO-responsive hematopoietic cell lines, they are differentially regulated and have selective effects on EPO signaling.

Whereas others have surveyed SOCS expression in response to other cytokines, we have demonstrated the relative stabilities of SOCS transcripts and examined the effects of signaling pathways other than Jak-Stat on SOCS gene induction. Through two independent approaches, one involving specific pathway suppression with either pharmacologic inhibitors or with dominant negative Stat5, and the other involving cell lines that activate only subsets of signaling pathways in response to EPO, we have provided solid evidence for the critical role of Stat5 in EPO-inducible SOCS1, SOCS2, SOCS3, and CIS transcriptional activation.

Our studies of the relative stabilities and EPO dependence of different SOCS transcripts reveal that all SOCS transcripts, except for SOCS2, are short-lived, with a half-life of less than 1 h. As SOCS protein levels may parallel transcript levels,[34–36] this short half-life provides a mechanism to explain how transient induction of SOCS gene expression can contribute to stringent control of SOCS protein levels and, therefore, effects on cytokine responsiveness. One group has proposed that because SOCS2 transcripts tend to persist on prolactin stimulation, whereas SOCS1 and SOCS3 appear only transiently, SOCS2 may serve to restore the sensitivity of cells to prolactin.[37]

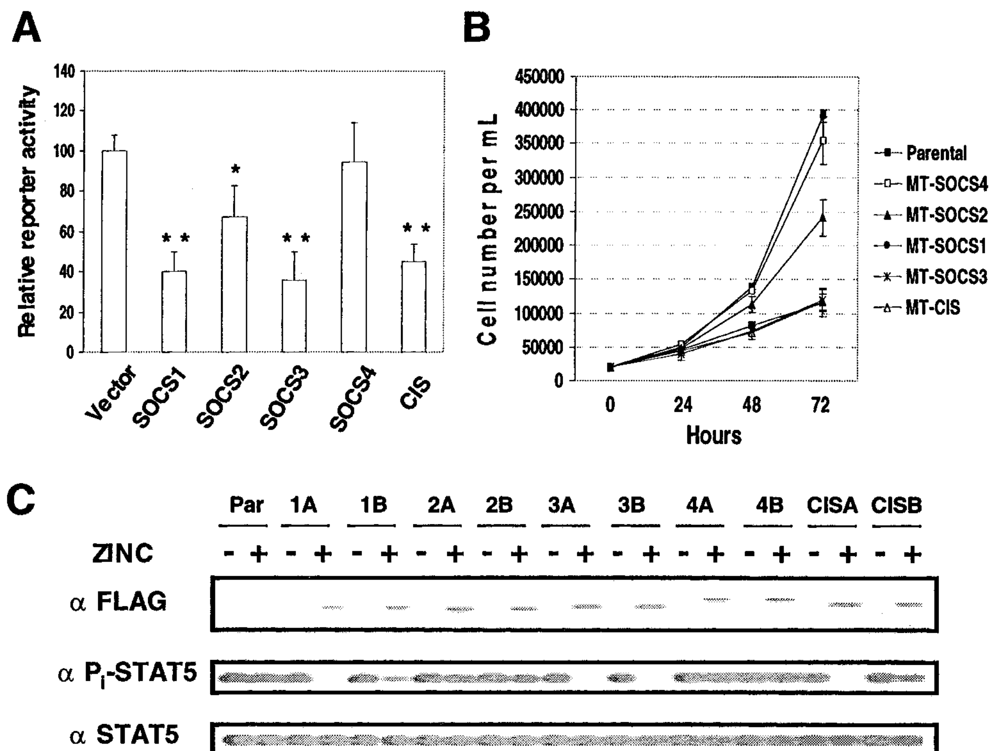

FIG. 4. Effects of transient overexpression of various SOCS family members on EPO-dependent (**A**) SOCS1 promoter activity, (**B**) cell proliferation, and (**C**) Stat5 phosphorylation. (**A**) 32D-EpoR cells, continuously cultivated in 1 U/ml EPO, were transiently cotransfected with a firefly luciferase reporter plasmid containing the SOCS1 promoter,[15] a Renilla luciferase plasmid for normalizing transfection efficiency, and either pEF vector or pEF constructs allowing expression of SOCS1, SOCS2, SOCS3, SOCS4, or CIS. Protein was extracted 36 h posttransfection and assayed for luciferase activity. Results are based on four independent experiments. Statistical analysis was performed using single factor ANOVA. $^{*}p < 0.05$; $^{**}p < 0.005$. (**B**) Per construct, two 32D-EpoR clones (A and B) that inducibly overexpress SOCS family members were plated at 20,000 cells/ml in medium containing 1 U/ml EPO and 30 μM zinc sulfate. Live cells (excluding trypan blue) were counted on each day, and results are based on analyses of two different clones per sample. In the absence of added zinc, EPO-dependent growth was similar among samples (data not shown). (**C**) The same clones described in Figure **B** were passaged continuously in EPO in the absence (−) or presence (+) of zinc sulfate (30 μM) for 24 h. Protein was isolated and subjected to Western analysis. The antibodies used recognize the FLAG epitope (top), phospho-Stat5 (middle), or both phosphorylated and unphosphorylated Stat5 (bottom). Par, parental cell line control; 1, pMT-SOCS1; 2, pMT-SOCS2; 3, pMT-SOCS3; 4, pMT-SOCS4.

Greater transcript stability, as we have demonstrated, can explain why SOCS2 levels are maintained after SOCS1 and SOCS3 levels decline in response to the same cytokine stimulus.

Furthermore, our data suggest that SOCS3 and SOCS4, unlike their family members, can be transcribed in the absence of EPO. Indeed, SOCS4 expression does not appear at all to depend on EPO. SOCS3, on the other hand, can be induced in response to EPO, although some transcription occurs in the absence of EPO, as demonstrated in this study. Consistently, SOCS3 expression can be detected in EpoR-deficient, Jak2-deficient, and Stat5-deficient mouse fetal livers.[20] Two possible explanations for EPO-independent SOCS3 transcription are (1) that SOCS3 expression is developmentally regulated in the absence of extracellular signals and (2) that another signaling pathway is involved. Because HCD57 is a cell line derived from a mouse infected at birth with Friend murine leukemia virus and 32D is derived from adult mouse bone marrow, our results show that EPO-independent transcription can occur even in nonembryonic cells. Further, they favor the hypothesis that EPO-independent SOCS3 transcription might be regulated by other yet unidentified factor(s).

To address the function of SOCS in EPO signaling, we have tested three readouts of EPO-mediated events, namely, activation of an EPO-dependent luciferase reporter, Stat5 phosphorylation, and cell proliferation, all of which concurred. That is, SOCS1, SOCS3, and CIS are the most potent inhibitors of EPO signaling, whereas SOCS2 is less potent and SOCS4 is ineffective. It is interesting that SOCS1 or CIS has as great an effect as SOCS3, given the fact that only the latter has been implicated in regulating erythropoiesis *in vivo.* Of course, overexpression studies have the disadvantage of overestimating the range of action of a given protein. However, overexpressing SOCS2 or SOCS4 did not comparably suppress EPO-mediated events. The fact that SOCS4 is neither induced by EPO nor affects EPO signaling when overexpressed distinguishes it from its family members. Thus, we have systematically analyzed the roles of distinct SOCS family members in

EPO signaling. Our results suggest that certain family members may have overlapping or redundant roles, and it would be informative to generate mice lacking multiple members of this family to further dissect their functions *in vivo*.

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