Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130

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Suppressor of cytokine signaling-3 (SOCS-3) is one member of a family of intracellular inhibitors of signaling pathways initiated by cytokines that use, among others, the common receptor subunit gp130. The SH2 domain of SOCS-3 has been shown to be essential for this inhibitory activity, and we have used a quantitative binding analysis of SOCS-3 to synthetic phosphopeptides to map the potential sites of interaction of SOCS-3 with different components of the gp130 signaling pathway. The only high-affinity ligand found corresponded to the region of gp130 centered around phosphotyrosine-757 (pY757), previously shown to be a docking site for the tyrosine phosphatase SHP-2. By contrast, phosphopeptides corresponding to other regions within gp130, Janus kinase, or signal transducer and activator of transcription proteins bound to SOCS-3 with weak or undetectable affinity. The significance of pY757 in gp130 as a biologically relevant SOCS-3 docking site was investigated by using transfected 293T fibroblasts. Although SOCS-3 inhibited signaling in cells transfected with a chimeric receptor containing the wild-type gp130 intracellular domain, inhibition was considerably impaired for a receptor carrying a Y→F point mutation at residue 757. Taken together, these data suggest that the mechanism by which SOCS-3 inhibits the gp130 signaling pathway depends on recruitment to the phosphorylated gp130 receptor, and that some of the negative regulatory roles previously attributed to the phosphatase SHP-2 might in fact be caused by the action of SOCS-3.

Cytokines control a wide variety of biological responses, and the duration and intensity of their effects must be tightly regulated. On stimulation by cytokine, specific cell-surface receptors oligomerize and cause activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (1). The transient nature of this signaling cascade is partly a consequence of the subsequent induction and/or activation of negative regulatory molecules such as SHP-1, protein inhibitor of activated STAT-3 (PIAS-3), and the suppressor of cytokine signaling (SOCS) proteins, each of which inhibit the JAK/STAT signaling pathway and ensure the appropriate level of response to a particular cytokine stimulus is maintained (2).

The SOCS family of proteins comprises eight members, SOCS-1 through SOCS-7 and cytokine-inducible SH2-containing protein (CIS), and the expression of several of these is known to be induced by cytokines (2, 3). Each of these proteins contains two regions of homology—a central SH2 domain and a C-terminal 40-aa motif known as the SOCS box. Although the SOCS box acts to recruit elongins BC, a protein complex implicated in the proteasomal degradation pathway (4, 5), the SH2 domains of the SOCS proteins are responsible for specific binding to activated (phosphorylated) signaling molecules and may also play a role in the mechanism of signal suppression.

A number of studies have identified cytokines that can induce the expression of SOCS-3 mRNA, including ciliary neurotrophic factor (6), leukemia inhibitory factor (7), IL-2 (8), IL-6 (9), IL-11 (10), leptin (11), prolactin (12), and growth hormone (13). Overexpression of SOCS-3 results in the inhibition of signaling by each of these cytokines, and under these conditions, SOCS-3 has been shown to associate physically with either JAK (14) or the growth hormone (15) and IL-2Rβ (8) receptors. However, given that overexpression can lead to elevated protein levels that result in nonspecific interactions, it is difficult to assess whether all of these observations are biologically relevant. Alternatively, gene knockout studies have shown that SOCS-3$^{-/-}$ mice die embryonically from a disease possibly associated with excessive fetal erythropoiesis (16).

Recently, it was proposed that the mechanism by which SOCS-3 inhibits signaling is identical to that of SOCS-1. As had been demonstrated for SOCS-1 (17), SOCS-3 was shown to associate with JAK2 in intact cells and to a synthetic phosphopeptide encompassing the activation loop from JAK2 (14). The region of SOCS-3 immediately N-terminal to the SH2 domain has also been shown to be important for biological activity (14, 18, 19) and, based on sequence similarity to SOCS-1, was proposed to function as a kinase active site inhibitor. However, despite these similarities, there is evidence to suggest that the mechanism of signaling suppression used by SOCS-3 differs from that of SOCS-1. Unlike SOCS-1, SOCS-3 does not inhibit the catalytic activity of JAK1 or JAK2 in an *in vitro* kinase reaction (19). Furthermore, the kinetics of IL-6 signal suppression, as measured by inhibition of STAT3 phosphorylation, is considerably slower for SOCS-3 compared with SOCS-1 (20). It has been proposed that these differences are the result of a weaker affinity of SOCS-3 for JAK relative to SOCS-1 (14). However, another possible explanation is that the primary binding target for SOCS-3 is not JAK, but other molecules within the signaling cascade such as the phosphorylated cytokine receptors or STAT proteins.

In an effort to define the mechanism by which SOCS-3 inhibits the gp130 signaling pathway, we sought to identify the molecular targets of SOCS-3 within this pathway and to quantify the affinities of these interactions. We demonstrate that the preferred high affinity-binding site for SOCS-3 is centered around the SHP-2-binding site at phosphotyrosine-757 of gp130, and that suppression of gp130 signaling by SOCS-3, but not SOCS-1, is impaired if this residue is mutated to phenylalanine.

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Abbreviations: EPOR, erythropoietin receptor; hEPO, human erythropoietin; JAK, Janus kinase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; pY757, phosphotyrosine-757; β-gal, β-galactosidase.

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Materials and Methods

SOCS-3 Protein Expression. Murine SOCS-3 cDNA was subcloned into a pET15b vector (Novagen) and expressed as a hexahistidine tagged protein in BL21 DE3 pLysS *Escherichia coli* (Stratagene). Recombinant protein was purified from cells lysed with 7 M guanidinium hydrochloride by immobilized metal affinity chromatography and refolded by dialysis into PBS containing 2 mM DTT, 2 mM EDTA, and 0.02% Tween 20. Refolded material was purified to homogeneity by ion exchange chromatography on a Mono S column (Pharmacia). Recombinant SOCS-3 was characterized by SDS/PAGE analysis and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The concentration of a stock solution of SOCS-3 used for quantitative binding analyses was determined by amino acid analysis.

Peptide Synthesis. All synthetic peptides were synthesized by using Fmoc amino acids activated with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate and contained a C-terminal amide. Biotinylated peptides were prepared by coupling *d*-biotin to the N terminus of resin-bound peptides before cleavage and deprotection. Crude peptides were purified by RP-HPLC and characterized by MALDI mass spectrometry.

Qualitative Analysis of SOCS-3 Phosphopeptide Interactions. Biotinylated phosphopeptides were immobilized onto streptavidin-agarose resin (Pierce), and 20 μl of these resins was incubated for 1 h at room temperature with 1 ml of 50 μg/ml SOCS-3 in 10 mM Tris, pH 7.5, containing 0.1% Tween 20. After removal of the protein solution, resin was washed twice with 1 ml PBS/0.1% Tween 20 and bound protein eluted with 25 μl of SDS sample buffer containing 10 mM DTT. Samples were analyzed by SDS/PAGE on a 12% polyacrylamide gel.

Biosensor Analyses. Biotinylated phosphopeptides (1 μg/ml) were captured onto a streptavidin-coated Biosensor chip (SA5, Pharmacia) at a density of ≈200 response units (RUs). Binding of recombinant SOCS-3 was measured by injecting solutions of protein diluted into PBS, 0.1% Tween 20 over the chip at a flow rate of 15 μl/min. For kinetic analysis of SOCS-3 binding, samples of protein were diluted and immediately analyzed to minimize losses of protein because of nonspecific protein absorption. After each binding measurement, residual SOCS-3 was desorbed from the immobilized ligand by washing with 6 M guanidinium hydrochloride (pH 8.0), followed by PBS, 0.1% Tween 20. Binding profiles were analyzed by using BIAEVALUATION software Ver. 3.0 (Pharmacia). To correct for nonspecific binding, sensorgrams obtained for binding of SOCS-3 to the noncognate gp130(674–688) phosphopeptide were subtracted from those for binding to the gp130(750–764) phosphopeptide. The dissociation constant K_d was calculated from a Scatchard-type analysis of the equilibrium response measurements obtained at different concentrations of SOCS-3 (21).

For competitive binding assays for measuring the affinities of SOCS-3 ligands in solution, immobilized gp130 (750–764) phosphopeptide was exposed to a dilution series of nonbiotinylated peptide ligand incubated with a fixed subsaturating amount of SOCS-3 (≈100 nM) in PBS containing 0.1% Tween 20 and 0.5 mg/ml BSA. The level of bound protein was recorded at a fixed time point within the sensorgram and divided by the corresponding level of SOCS-3 bound to the peptide chip in the absence of competing ligand. These fractional binding values (f) were fitted to the equation $f = 1/(1 + (c/IC_{50})^m)$, where c = the concentration of soluble SOCS-3 ligand, m = the curvature constant, and IC_{50} values correspond to the concentration of ligand required to displace 50% of the bound SOCS-3.

Receptor Expression Constructs. An Asc I–Mlu I cDNA fragment containing the mature coding region of mouse erythropoietin receptor (EPOR) was cloned in-frame with the IL-3 signal and FLAG sequences of pEF-FLAG-S. A silent mutation was introduced at nucleotide 771 to create a unique *Bam*HI site, and PCR-derived DNA fragments encoding the mouse gp130 transmembrane and cytoplasmic domain (amino acids 618–917) were then cloned in-frame with the mouse EPOR extracellular domain (amino acids 25–249; EPOR/gp130). The Y757F mutation was introduced by using overlap PCR (EPOR/gp130Y757F). All constructs were sequenced in their entirety before use.

Luciferase Assays. Generation of the expression vectors for SOCS-1 and SOCS-3 and luciferase assays have been described previously (19). Briefly, 293T cells were transiently transfected with receptor and SOCS expression plasmids, a leukemia inhibitory factor-responsive reporter construct [APRE-luc (22)], and a β-galactosidase (β-gal) reporter construct under a constitutive promoter [Srα-β-gal (23)]. Cells were incubated overnight with 10 units/ml recombinant human erythropoietin (hEPO) and lysed. Luciferase activity from triplicate samples was determined and normalized against β-gal activity.

Results

Identification of Phosphotyrosine-757 (pY757) in gp130 as a Potential SOCS-3 Interaction Site. To investigate whether SOCS-3 might interact with tyrosine-phosphorylated sites within gp130 or relevant STAT proteins, we prepared phosphopeptides corresponding to regions surrounding each of the gp130 cytoplasmic tyrosine residues, in addition to peptides based on the known tyrosine phosphorylation sites within STAT1 and STAT3 (Fig. 1*A*). These peptides contained an N-terminal biotin moiety and were immobilized onto streptavidin–agarose resin. Recombinant SOCS-3 was incubated with peptide resin, and after washing, any bound protein was eluted with SDS sample buffer. SDS/PAGE analysis showed that only the peptide centered around the pY757 of gp130 was able to capture SOCS-3 efficiently (Fig. 1*B*).

The biotinylated form of gp130(750–764) phosphopeptide was captured onto a streptavidin Biosensor chip, and binding of recombinant SOCS-3 was measured. As a nonspecific control, the phosphorylated gp130(674–688) peptide was captured on a separate channel of the same chip. Concentration-dependent binding of SOCS-3 to the immobilized gp130(750–764) phosphopeptide was observed in the range of 8.65–1,110 nM (Fig. 2*A*), whereas no direct binding was detected to the gp130(674–688) phosphopeptide. The dissociation constant for peptide binding was calculated from a Scatchard analysis of the equilibrium response values at different concentrations of SOCS-3 (Fig. 2*B*). The affinity of this interaction (K_d = 42 nM) is high and characteristic of the binding constants observed for other SH2 domains binding to high-affinity phosphopeptide ligands (24).

To ascertain whether SOCS-3 binding depended on the phosphorylation state of the peptide, a competitive inhibition-binding assay was developed by using the Biosensor instrument. A subsaturating amount of SOCS-3 was incubated with different dilutions of a soluble competing peptide, and this mixture was injected onto the sensorchip containing the immobilized gp130(750–764) phosphopeptide and relative binding measured. Although an IC_{50} value of 110 nM was obtained for the phosphorylated gp130(750–764) peptide, the nonphosphorylated analogue bound with an IC_{50} of 2.1 mM, a 19,000-fold lower binding affinity (Fig. 3*A*). We also used this competition assay to reassess the potential solution binding of the phosphopeptides listed in Fig. 1. With the exception of the biotinylated gp130(750–764) phosphopeptide, none of these peptides showed measurable binding to SOCS-3 at concentrations up to 100 μM (data not shown).

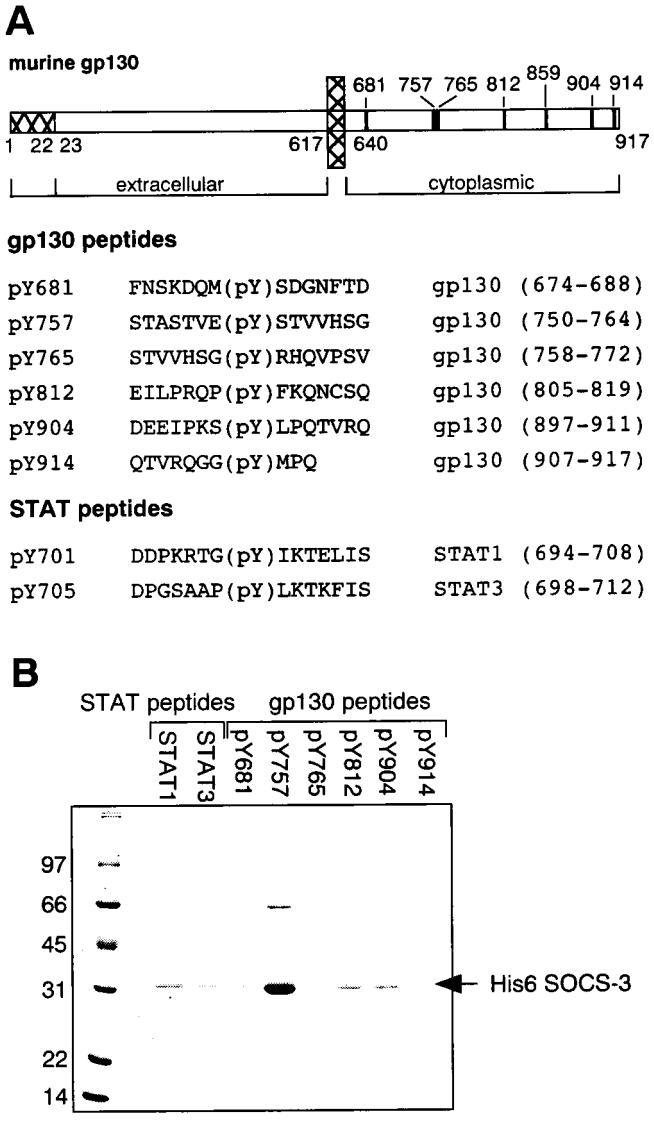

Fig. 1. A gp130-derived phosphopeptide interacts specifically with SOCS-3. (*A*) Biotinylated phosphopeptides were synthesized corresponding to the regions of murine gp130 surrounding each cytoplasmic tyrosine residue. The pY812 peptide contained a cysteine residue in the reduced form, whereas a pY859 peptide was not synthesized, as this tyrosine is not conserved in human gp130. Similarly, peptides corresponding to regions surrounding the known tyrosine phosphorylation sites in STAT1 and STAT3 were also synthesized. (*B*) Peptides shown in *A* were immobilized on streptavidin–agarose resin and incubated with recombinant SOCS-3. Only the gp130 pY757 peptide showed any significant binding of SOCS-3 as detected by Coomassie-stained SDS/PAGE analysis of the resin eluates.

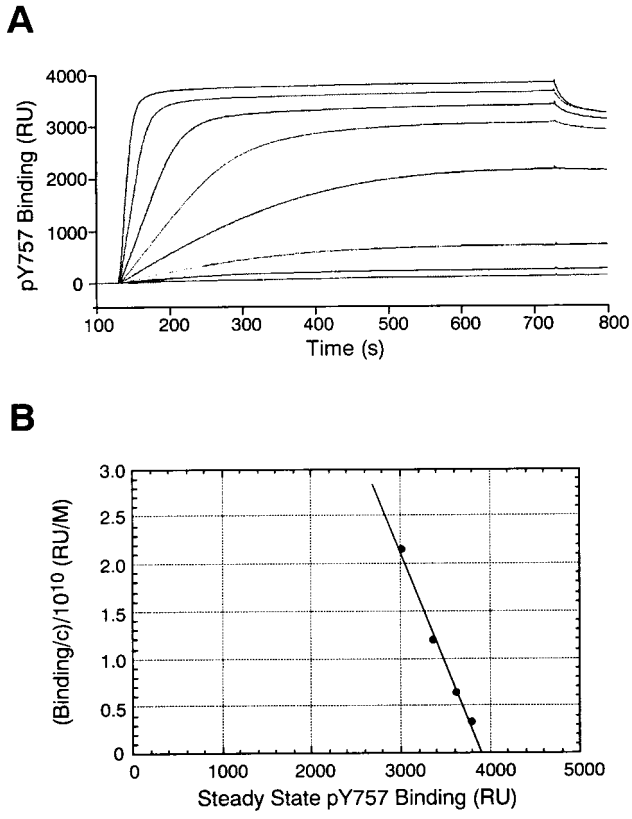

Fig. 2. Functional characterization of SOCS-3 protein. (*A*) Biosensor analysis of SOCS-3 binding to the gp130-derived phosphopeptide biotin-STASTVEpYS-TVVHSG. Sensorgrams correspond to a 2-fold serial dilution series of SOCS-3 (8.65–1,110 nM) binding to the immobilized peptide on a streptavidin sensorchip. (*B*) Determination of binding constants by Scatchard-type analysis. Data shown in *A* were used to calculate the association constant for SOCS-3 binding to the immobilized gp130 peptide. Plateau binding values at steady state (four highest concentrations of SOCS-3) were plotted against the ratio of plateau binding to SOCS-3 concentration (Binding/*c*). The association binding constant ($K_a = 2.4 \times 10^7\ M^{-1}$) was calculated from the slope of the plot of Binding/*c* vs. Binding. The dissociation constant (K_d) = $1/K_a$ = 42 nM.

SOCS-3 Binds Selectively to gp130 but Only Weakly to JAK Peptides. Earlier reports had demonstrated that endogenous SOCS-3 was able to associate with a phosphopeptide corresponding to the activation loop of the JAK2 domain (14). We therefore sought to make a quantitative comparison between the affinity of SOCS-3 for the gp130(750–764) phosphopeptide vs. phosphopeptides derived from the activation loops of JAKs 1–3 (Fig. 3*B*). The tightest-binding JAK phosphopeptide was that from JAK-3 with an IC_{50} value of 140 μM; however, this affinity was ≈1,000-fold weaker than that for the gp130(750–764) phosphopeptide. The JAK2-derived peptide bound SOCS-3 with even lower affinity, some 10,000-fold weaker than gp130(750–764). Based on these binding data, it seems far more likely that pY757 on gp130 is the physiological site of SOCS-3 interaction and not the activation loop on the JAKs.

SOCS-3 Recognizes an Extended Epitope on gp130. SH2 domains recognize tyrosine-phosphorylated polypeptides as ligands. Although a central phosphotyrosine residue is the primary binding determinant, other amino acids within the immediate vicinity of the phosphotyrosine residue also contribute to the specificity of the association. In general, two or three of the amino acids between the first (pY+1) and fifth (pY+5) residue downstream from phosphotyrosine contribute to SH2 binding, whereas upstream residues do not participate in binding interactions (25, 26).

To understand better the structural basis of SOCS-3 ligand-binding specificity, a series of gp130(750–764) phosphopeptide analogues were synthesized. These analogues either were truncated or contained single alanine substitutions relative to the gp130(750–764) parent phosphopeptide. Peptides truncated at the N terminus were acetylated to prevent any possibility of a charge repulsion effect altering binding to SOCS-3. The affinities of these peptides for SOCS-3 were measured in the competitive binding assay and compared with that of the gp130(750–764) phosphopeptide (Table 1). Based on the data from this series of peptides, the side chains of at least five nonphospho-

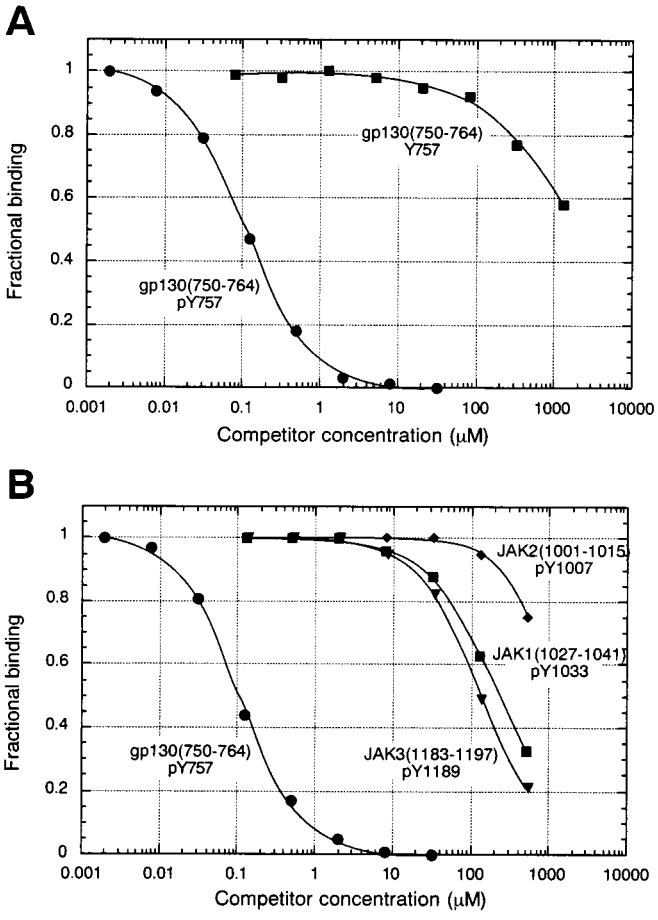

Fig. 3. Comparison of SOCS-3 affinity for phosphorylated vs. nonphosphorylated gp130 peptide and JAK-derived peptides. (*A*) Solution binding of phosphorylated and nonphosphorylated forms of gp130(750–764) peptide to SOCS-3 was measured in a competitive binding assay. The IC_{50}s for inhibition of SOCS-3 binding to immobilized ligand were 110 ± 2.7 nM for the phosphorylated peptide and 2.1 ± 0.2 mM for the unphosphorylated peptide. (*B*) Solution binding of JAK-derived peptides to SOCS-3. These phosphopeptides represent the activation loop sequences in JAK1, 2, and 3 and contain a phosphotyrosine residue corresponding to the autophosphorylation site. The amino acid sequences of these peptides were JAK1: IETDKE(pY)YTVKDDRD, JAK2: LPQDKE(pY)YKVKEPGE, JAK3: LPLGKD(pY)YVVREPGQ. The IC_{50} values for these peptides were: JAK1, 230 ± 6.6 μM; JAK2, 1,200 ± 50 μM; JAK3, 140 ± 4.8 μM. The IC_{50} value for the gp130(750–764) phosphopeptide, measured in the same experiment, was 110 ± 4.6 nM

tyrosine residues appear to be interacting with SOCS-3, these being the pY-1 and -2 residues, in addition to pY+3, +4, and + 5. Although it is possible that the pY+1 and +2 side chains also participate in interactions with SOCS-3, this is not apparent from the alanine substitution data. Thus, compared with most other SH2 domains, SOCS-3 recognizes an extended polypeptide epitope, and this interaction includes ligand residues that are N- and C-terminal to phosphotyrosine.

pY757 Is Important for Inhibition of gp130 Signaling by SOCS-3. The peptide studies indicated that Y757 was likely to have an important role in the specificity and mechanism of SOCS-3 action. It was therefore important to confirm these findings by using a biological assay. A chimeric receptor was created that contained the EPOR extracellular domain and the gp130 cytoplasmic domain (EPOR/gp130). A second receptor was created in which Y757 had been mutated to phenylalanine (EPOR/gp130Y757F). 293T cells were transiently transfected with cDNAs expressing receptor constructs and either SOCS-3 or SOCS-1 in the presence of reporter genes. Cells were then incubated overnight with hEPO, and luciferase activity was determined. Mutation of Y757 in the gp130 cytoplasmic domain has been reported to result in a hyperactive receptor complex, presumably from the loss of a negative regulator such as SHP-2 (27). Consistent with this, activation of the EPOR/gp130Y757F receptor resulted in a 4-fold higher level of luciferase activity than activation of the EPOR/gp130 receptor (Fig. 4). Higher concentrations of SOCS-3 DNA were required to inhibit the EPOR/gp130Y757F receptor than the EPOR/gp130 receptor in which Y757 had not been mutated (50% inhibition at 50 ng compared with 4 ng SOCS-3 DNA; Fig. 4*D*). This difference was not observed when SOCS-1 DNA was used (Fig. 4*A*) and strongly suggests that SOCS-3 acts by binding to Y757 on gp130.

Table 1. Binding of gp130(750–764) phosphopeptide analogues

Peptide	IC_{50}, nM	IC_{50} analogue/ IC_{50} wild type
STASTVE(pY)STVVHSG	110	
STASTVE(pY)ATVVHSG	72	0.65
STASTVE(pY)SAVVHSG	110	1.0
STASTVE(pY)STAVHSG	3,400	31
STASTVE(pY)STVAHSG	1,100	10
STASTVE(pY)STVVASG	770	7.0
STASTVE Y STVVHSG	2.1×10^6	19,000
STASTVE(pY)STVVHS	61	0.55
STASTVE(pY)STVVH	77	0.70
STASTVE(pY)STVV	1,100	10
Ac-TVE(pY)STVVHSG	120	1.1
Ac-VE(pY)STVVHSG	110	1.0
Ac-E(pY)STVVHSG	970	8.8
Ac-(pY)STVVHSG	9,600	87
Ac-AE(pY)STVVHSG	580	5.3
Ac-VA(pY)STVVHSG	550	5.0

Ac, acetyl; pY, phosphotyrosine.

Discussion

Both SOCS-1 and SOCS-3 are potent inhibitors of the biological effects mediated by the IL-6 family of cytokines, whereas other SOCS proteins, such as CIS or SOCS-2, do not inhibit this signaling pathway (19). Constitutive overexpression of either SOCS-1 or SOCS-3 results in the suppression of STAT3 phosphorylation, indicating that these proteins act as inhibitors of the JAK/STAT signaling pathway. However, differences in their ability to inhibit JAK activity (19) and the rates at which they suppress STAT3 phosphorylation (20) suggest that the mechanism of signaling inhibition by SOCS-1 and SOCS-3 may be different.

We therefore investigated whether SOCS-3, expressed as a recombinant protein, was capable of interacting with other components of the gp130 signaling pathway. When phosphopeptides encompassing each of the tyrosine residues on gp130 or the known tyrosine phosphorylation sites in STAT1 and STAT3 were immobilized onto streptavidin–agarose, only one phosphopeptide, that spanning the region around Y757 on mouse gp130, was found to associate with SOCS-3 to any appreciable degree. Quantitative analysis revealed that this was a high-affinity interaction depending on phosphorylation of the central tyrosine residue in the ligand (Y757). Phosphopeptides derived from the activation loops of JAK1, 2, and 3, previously reported as the target site for SOCS-3 association *in vivo*, bound to SOCS-3 1,000- to 10,000-fold more weakly than the high-affinity gp130 peptide. Moreover, the ability of SOCS-3 to inhibit

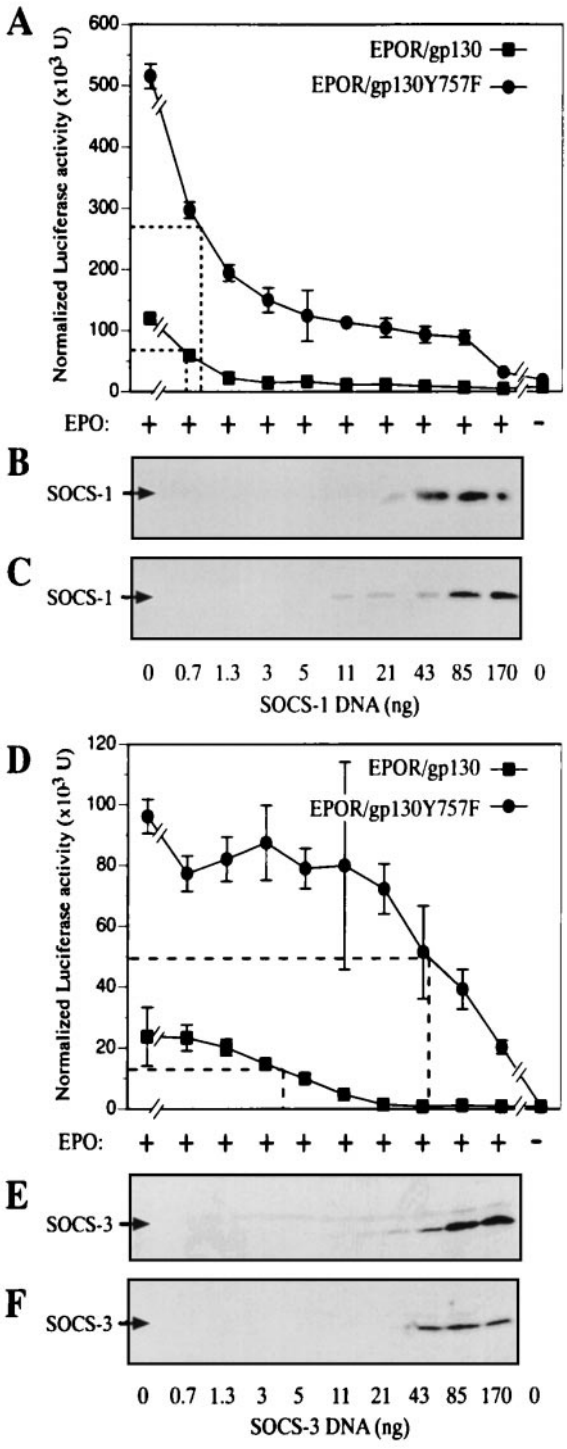

Fig. 4. SOCS-3, but not SOCS-1, inhibition of gp130 signaling is mediated through gp130 Y757. (*A*) 293T cells were transiently transfected with cDNAs expressing SOCS-1 and either EPOR/gp130 or EPOR/gp130Y757F in the presence of the APRE-luc and Srα-β-gal reporter genes. Cells were incubated in the presence (+) or absence (−) of 10 units/ml hEPO overnight and cell extracts prepared. Luciferase activity from triplicate samples was determined and normalized against β-gal activity. (*B*) SOCS-1 protein levels in 293T cells expressing EPOR/gp130 were determined by Western blot with anti-Flag antibody. (*C*) SOCS-1 protein levels in 293T cells expressing EPOR/gp130Y757F. (*D*) 293T cells were transiently transfected with cDNAs expressing SOCS-3 and either EPOR/gp130 or EPOR/gp130Y757F in the presence of the APRE-luc and Srα-β-gal reporter genes. (*E*) SOCS-3 protein levels in 293T cells expressing EPOR/gp130 were determined by Western blot with anti-Flag antibody. (*F*) SOCS-3 protein levels in 293T cells expressing EPOR/gp130Y757F.

signaling by a chimeric gp130 receptor was impaired when Y757 was mutated to phenylalanine, suggesting that recruitment of SOCS-3 to gp130 is relevant to the mechanism of inhibition. By contrast, SOCS-1 inhibited wild-type and mutant forms of the receptor equally well, indicating that SOCS-1 must function in a way that is distinct from that of SOCS-3.

Although the data presented here suggest that gp130 is a far more likely binding target of SOCS-3 than JAK, our studies with synthetic phosphopeptide ligands have addressed only potential interaction partners of the SH2 domain and not the N-terminal region of SOCS-3. The regions of SOCS-3 and SOCS-1 upstream of the SH2 domain have been suggested to resemble the activation loop sequences of the JAKs and may function as active-site inhibitors in the same way that the activation loop inhibits catalytic activity before its phosphorylation (14, 17). Although the localization of SOCS-3 to gp130 might raise questions as to how the N-terminal region could bind simultaneously to the active site of JAK, it should be noted that Y757 on gp130 is phosphorylated in response to cytokine stimulation (28) and is therefore likely to be in close proximity to JAK.

Tyrosine 757 on gp130 has been characterized previously as the docking site for another regulator of cytokine signaling, the tyrosine phosphatase SHP-2, which binds to activated gp130 via its N-terminal SH2 domain (27–29). SHP-2 can regulate gp130 signaling positively through activation of the mitogen-activated protein kinase (MAPK) pathway (29), although the precise mechanism by which SHP-2 couples gp130 signaling to the MAPK pathway remains unresolved. In this regard, SOCS-3 could also act to suppress MAPK signaling by competing with SHP-2 for binding to the pY757 docking site.

In contrast to this positive regulatory role, SHP-2 has also been reported to act as a negative regulator of gp130 signaling (27, 30, 31). Much of the data that has been used in support of a negative regulatory role for SHP-2 is based on observed increases in gp130-dependent signaling when Y757 is mutated. Clearly, inhibition of gp130 signaling by SOCS-3 would also be adversely affected by mutation of Y757. Recently, a human gp130 mutant with a Y759 (equivalent to Y757 in the mouse) to phenylalanine mutation has been used to replace the endogenous gp130 genes in mice (32). These mice displayed lymphoadenopathy, splenomegaly, high Ig and T-helper 1 cytokine levels, and an enhanced acute-phase response. These data suggest that this site on gp130 mediates important negative regulation of lymphoid cell production and actions, and it now remains to be determined whether these are mediated by SOCS-3, SHP-2, or a combination of the two.

Recognition of the same site on gp130 by both SOCS-3 and SHP-2 implies that these two SH2-containing proteins share an overlapping specificity for phosphotyrosine-containing ligands. The unusual preference of SOCS-3 for a ligand containing a valine residue at pY-2 is a feature also shared by the SHP-2 N-terminal SH2 domain and has been attributed to substitution of the conserved αA2 residue within the SH2 domains of SHP-2 (33). This residue is an arginine in most SH2 domains but is glycine in SHP-2 and also in SOCS-3 (SOCS-3 residue 53). In addition, the binding interface between SHP-2 and a phosphotyrosine-containing ligand extends out to the pY+5 residue and is characterized by a hydrophobic residue such as valine at the pY+3 position (34, 35), much as we observe for SOCS-3. In light of this, it is possible that SOCS-3 might recognize other SHP-2-binding sites on activated signaling molecules, particularly for those pathways where both negative regulators have been implicated. One such example is signaling via the leptin receptor. The leptin receptor is related to gp130, and leptin induces the expression of SOCS-3, which in turn attenuates signaling (11). SHP-2 has also been identified as a negative regulator of leptin, and this activity depends on association with the leptin receptor at a VK(pY)ATLIS

motif (36), which would not be inconsistent with a potential binding site for SOCS-3. To date, no studies have tested this possibility.

To our knowledge, these studies represent the first example of a quantitative assessment of a SOCS protein binding to potential interaction partners. This led us to identify a unique high-affinity SOCS-3 binding site on gp130, which, if mutated, affects the ability of SOCS-3 to inhibit gp130 signaling. Thus, although SOCS-1 and SOCS-3 have overlapping activities as inhibitors of cytokine signaling, they appear to do so via a distinct biochemical mechanism.

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