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Intracellular Delivery of a Cell-Penetrating SOCS1 that Targets IFN- γ Signaling

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Abstract

Suppressor of cytokine signaling–1 (SOCS1) is an intracellular inhibitor of the Janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway that couples interferon- γ (IFN- γ) signaling to the nucleus. Because several inflammatory diseases are associated with uncontrolled IFN- γ signaling, we engineered a recombinant cell-penetrating SOCS1 (CP-SOCS1) to target this pathway. Here, we show that CP-SOCS1, analogous to endogenous SOCS1, interacted with components of the IFN- γ signaling complex and functionally attenuated the phosphorylation of STAT1, which resulted in the subsequent inhibition of the production of proinflammatory chemokines and cytokines. Thus, controlled, intracellular delivery of recombinant CP-SOCS1 boosted the anti-inflammatory potential of the cell by restoring the homeostatic balance between pro- and anti-inflammatory signaling. This approach to controlling signal transduction has potential use for therapeutic targeting of signaling pathways associated with inflammatory diseases.

Introduction

Cytokines are mediators of the host immune response, and, as such, a delicate balance must be maintained between their physiologic role in innate and adaptive immunity and pathologic hyperresponsiveness. The latter can lead to the production of a cytokine storm, which is a hallmark of the potentially lethal acute inflammatory responses that underlie sepsis and septic shock. Additionally, chronic autoimmune disorders, such as inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis, are linked to the uncontrolled production of proinflammatory cytokines. Interferon- γ (IFN- γ) is a pleiotropic, proinflammatory cytokine that induces the production of a spectrum of mediators of inflammation and apoptosis (1). IFN- γ signaling is primarily mediated through the receptor-recruited Janus kinases (JAKs) and their substrates, the signal transducer and activator of transcription (STAT) proteins (2). The binding of IFN- γ to the cognate receptor (IFN- γ R) promotes its oligomerization and activation of JAKs to phosphorylate specific tyrosine residues in the cytoplasmic domain of IFN- γ R, which provide docking sites for Src homology 2 (SH2) domain-containing signal transducers (3). Among the candidates that can interact with these docking site(s), STAT proteins are the most important for transducing cytokine signaling to the nucleus (2,4–6). This signaling culminates in expression of genes that encode proinflammatory chemokines and cytokines, as well as the anti-inflammatory physiologic regulators of inflammation known as suppressors of cytokine signaling (SOCS). Thus, the members of the family of inducible SOCS proteins are classic negative feedback inhibitors of the IFN- γ -induced JAK–STAT pathway that counteract potentially noxious intracellular signaling induced by excessive inflammation (7–10).

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The SOCS family is composed of eight SH2 domain-containing proteins: cytokine-inducible SH2 (CIS) and SOCS1 to SOCS7. Each protein uniquely disrupts signaling by either inhibiting the kinase activities of JAKs or interacting with activated cytokine receptors (10–14). In addition, SOCS proteins contain a C-terminal SOCS box that associates with cullin and elongins B and C to form a ubiquitin ligase that targets proteins or signaling complexes for proteasomal degradation (15). Among the SOCS family members, SOCS1 and SOCS3 are the best characterized in terms of their abilities to regulate cytokine signaling. Although structurally similar to SOCS3, SOCS1 inhibits cytokine signaling by binding directly to JAK, whereas SOCS3 inhibits JAK only in the presence of gp130 (glycoprotein 130) (12,16,17). In addition, SOCS1 binds directly to the IFN- γ R, thus promoting potent inhibition of IFN- γ signaling even at low abundance (14,18,19). As such, SOCS1 is a more potent inhibitor of IFN- γ signaling than is SOCS3 (12,20).

The prominence of SOCS1 in extinguishing cytokine signaling is evident from the phenotype of SOCS1-deficient mice; they die within 3 weeks of the neonatal period from severe inflammatory complications that damage multiple organs. This overwhelming and relentless inflammation can be rescued by blocking IFN- γ signaling (21–25). IFN- γ primarily activates STAT1, a key intracellular mediator of the expression of genes encoding proinflammatory cytokines, which indicates the importance of SOCS1 in preventing hyperactivation of this transactivator. Consequently, *Socs1*^{-/-}*Stat1*^{-/-} animals exhibit a phenotype of grossly reduced inflammation identical to that of *Socs1*^{-/-}*Ifn- γ* ^{-/-} mice, confirming the role of IFN- γ and STAT1 in the lethal inflammatory disease associated with SOCS1 deficiency (14).

In addition, *Socs1*^{-/-}*Ifn- γ* ^{-/-} and *Socs1*^{-/-}*Stat1*^{-/-} mice are hypersensitive to lipopolysaccharide (LPS)-induced lethal shock, which is suggestive of a role for SOCS1 in the inhibition of signaling evoked by LPS, the most potent inducer of inflammation known (26, 27). Indeed, SOCS1 plays an important role in quenching the activation of Toll-like receptor (TLR) signaling by LPS (28,29). SOCS1 binds to myeloid differentiation marker 88 (MyD88) adaptor-like protein (MAL, also known as TIRAP) after its phosphorylation by Bruton's tyrosine kinase (Btk) and induces the ubiquitination and degradation of MAL. Thus, MAL-dependent phosphorylation and transactivation of nuclear factor κ B (NF- κ B) p65 (also known as RelA) is also attenuated by SOCS1, thereby silencing the transcription of NF- κ B-dependent genes (29,30). In addition, SOCS1 inhibits the expression of the gene that encodes CD40 (31). This finding is noteworthy because aberrant CD40 protein level is associated with human diseases such as multiple sclerosis, rheumatoid arthritis, and Alzheimer's disease (31). Together, these advances indicate that SOCS1 is a multifaceted inhibitor of proinflammatory signaling that underlies the production of chemokines and cytokines in acute and chronic inflammatory and autoimmune disorders. Implicit in the uncontrolled progression of these diseases are insufficient amounts of SOCS1 and other physiologic regulators of proinflammatory signaling.

Until recently, intracellular targeting of specific signaling pathways has relied on the forced expression of genes that encode intracellular signal transducers and their regulators. This approach has provided valuable information about the mechanism of intracellular action of these molecules, even though it is subject to the vagaries of the variable efficiency of transfection and an inability to control the abundance of the proteins produced. To overcome these obstacles, we designed a method of intracellular delivery of proteins, a technique that incorporates the attachment of a cell-penetrating (CP) membrane translocation motif (MTM) derived from the hydrophobic region of a signal sequence to a physiologic intracellular anti-inflammatory protein. With this facile platform, we developed recombinant CP-SOCS1 for the controlled delivery (in terms of time and concentration) of physiologic suppressors of cytokine signaling to probe and block IFN- γ -evoked signal transduction. We show that engineered CP-SOCS1 reached the cell interior and that it targeted components of the IFN- γ signaling complex,

thereby attenuating IFN- γ -induced phosphorylation of STAT1 and production of proinflammatory cytokines and chemokines in established and primary lines of macrophages. Thus, the development and functional characterization of CP-SOCS1 indicates its utility for restoring the physiologic anti-inflammatory potential of cells involved in the production of excessive amounts of cytokines, chemokines, and other mediators of inflammation. In addition, our work provides evidence of the applicability of intracellular delivery of proteins as a means to identify previously unrecognized protein interacting partners under steady-state conditions rather than under uncontrolled, forced expression, which is prone to the potentially artifactual overproduction of intracellular recombinant proteins with uncontrolled stoichiometry of analyzed protein-protein interactions.

Results

Engineering of a recombinant, cell-penetrating SOCS1 protein in *Escherichia coli*

We have previously shown that a CP form of SOCS3 produced in the *E. coli* expression system is effective in reducing inflammation and apoptosis in vivo (32). However, SOCS1 has greater anti-inflammatory capabilities than does SOCS3, which is manifested in SOCS1 primarily targeting STAT1, whereas SOCS3 targets STAT3 (12,29–31). Especially relevant is the phenotype of mice deficient in *Socs1*, which includes rampant inflammation of multiple organs mediated by endogenous IFN- γ , while the expression of *Socs3* is maintained (23). Therefore, we designed a series of recombinant CP and non-CP forms of murine SOCS1 in an attempt to target the IFN- γ -induced signaling pathway. We also constructed deletion mutants of CP-SOCS1 that lacked either the proline, glutamic acid, serine, threonine (PEST) motif or both the PEST motif and the SOCS box, to establish whether these motifs were dispensable for the anti-inflammatory activity of CP-SOCS1. All proteins contained a polyhistidine tag to facilitate their purification by metal-affinity chromatography (Fig. 1). Cell-penetrating forms of SOCS1 contained a physiologic MTM derived from the hydrophobic signal sequence region of human fibroblast growth factor 4 (FGF4), which enables attached cargo to cross the plasma membrane (33). Recombinant mouse non-CP-SOCS1 and CP-SOCS1 proteins (containing an N- or C-terminal MTM) expressed as inclusion bodies (IBs) from *E. coli* were purified and reconstituted, and their purities and yields were similar (Fig. 2). The presence of contaminating LPS in recombinant proteins was analyzed by the *Limulus* assay, which usually reveals the presence of LPS at concentrations of 1 ng per microgram of recombinant protein. Therefore, for our experiments, we used LPS-hyporesponsive AMJ2.C8 macrophages (34) or bone marrow-derived macrophages (BMDMs) obtained from LPS-hyporesponsive C3H/HeJ mice to mitigate the potential effect of contamination of recombinant proteins by residual LPS.

Intracellular delivery of CP-SOCS1

We first compared the abilities of CP-SOCS1 and non-CP-SOCS1 proteins to traverse the cell membrane of LPS-hyporesponsive AMJ2.C8 macrophages. We based this experiment on a protease-accessibility assay and on the immunoprecipitation of internalized SOCS1. Cultured cells were treated with non-CP-SOCS1 or CP-SOCS1 proteins for 1 hour. Subsequently, the broad-range protease, proteinase K, was applied to remove SOCS1 proteins from the cell surface, thereby preventing contamination of the cell lysates used in the subsequent analysis by SOCS1. We used cells treated with the non-CP-SOCS1 protein and cells treated with diluent as negative controls. An isotype-matched antibody for cells treated with CP-SOCS1 provided an additional control for these experiments. We prepared lysates of cells treated with CP-SOCS1 and controls for immunoprecipitation with the indicated antibodies. Endogenous SOCS1 was not detected in diluent-treated cells (Fig. 3), consistent with previous reports that SOCS1 is undetectable unless induced by proinflammatory agonists (35). In contrast, an immunoreactive band consistent with the size of CP-SOCS1 was immunoprecipitated from lysates of cells treated with CP-SOCS1 by an antibody against SOCS1 (anti-SOCS1) (Fig. 3).

That this band was detected in samples treated with proteinase K indicated the intracellular location of CP-SOCS1 because it was not accessible to protease activity. The intracellular concentration of CP-SOCS1 in AMJ2.C8 cells, based on packed cell volume, was 11.6 nM (fig. S1).

Targeting of IFN- γ signaling pathway components and inhibition of IFN- γ -induced phosphorylation of STAT1 by CP-SOCS1

We next determined whether CP-SOCS1 delivered intracellularly could interact with components of the IFN- γ signaling pathway. We stimulated CP-SOCS1-pulsed AMJ2.C8 macrophages with IFN- γ and then performed Western blotting analysis of samples immunoprecipitated with antibodies against JAK2 (anti-JAK2) or STAT1 (anti-STAT1), which are interacting partners of SOCS1. These experiments revealed immunoreactive bands consistent with the size of CP-SOCS1 in samples immunoprecipitated with anti-JAK2 or anti-STAT1, indicating that recombinant CP-SOCS1 interacted with these components (Fig. 4, A and B). Endogenous SOCS1 was not detectable under these experimental conditions.

SOCS1 serves as a cytoplasmic feedback inhibitor of the tyrosine phosphorylation of STAT1, the primary transcription factor activated by IFN- γ (2,10). Because CP-SOCS1 interacted with the IFN- γ signaling complex, it was expected to also inhibit the phosphorylation of STAT1. To assess this, we analyzed the extent of phosphorylation of STAT1 after stimulation of CP-SOCS1-pulsed cells with IFN- γ and LPS. We observed concentration-dependent inhibition of STAT1 phosphorylation in AMJ2.C8 macrophages that contained CP-SOCS1 tagged with MTM at its N terminus (Fig. 5A). The concentration of CP-SOCS1 that inhibited phosphorylation of STAT1 by 50% (IC₅₀) was <1.9 μ M. CP-SOCS1 also attenuated IFN- γ -induced phosphorylation of STAT1 in BMDMs from C3H/HeJ mice (Fig. 5C). The inhibitory effect of CP-SOCS1 in both of these cell types was confirmed by Western blot analysis (Fig. 5, B and D). To exclude the possibility that the MTM tag was responsible for the observed decreased phosphorylation of STAT1 in CP-SOCS1-pulsed cells, we transfected HEK 293F cells with plasmids encoding non-CP-SOCS1 or CP-SOCS1, incubated the cells overnight, and analyzed the extent of STAT1 phosphorylation in response to a 15-min stimulation with IFN- γ . We observed at least a 50% reduction in the abundance of phosphorylated STAT1 in cells containing either non-CP-SOCS1 or CP-SOCS1 proteins compared to vector-transfected, control cells (fig. S2), confirming that the MTM in CP-SOCS1 was not responsible for inhibiting the phosphorylation of STAT1 in response to IFN- γ .

Next, we incubated AMJ2.C8 cells with non-CP-SOCS1 or CP-SOCS1 for 1 hour, after which the recombinant proteins were removed from the culture media (time 0) and the cells were subsequently stimulated with IFN- γ . As early as 20 min after the removal of CP-SOCS1 protein, the abundance of phosphorylated STAT1 in response to IFN- γ was similar in the cells pulsed with CP-SOCS1 and the control cells pulsed with non-CP-SOCS1 (fig. S3). These results indicated that the inhibitory effect of CP-SOCS1 was both short-lived and reversible. Together, these data suggested that the functions of CP-SOCS1 recapitulated those of endogenous SOCS1.

CP-SOCS1 inhibits IFN- γ -induced production of proinflammatory chemokines and cytokines

Inhibition of IFN- γ -induced phosphorylation of STAT1 by CP-SOCS1 should result in attenuation of the production of cytokines and chemokines in IFN- γ -stimulated AMJ2.C8 macrophages. To test this hypothesis, we analyzed the extent of the IFN- γ -stimulated production of cytokines and chemokines in cells incubated with CP-SOCS1. Pretreatment of AMJ2.C8 macrophages for 1 hour with CP-SOCS1 was effective in reducing the production of the chemokines CXCL10 (also known as IP-10) and RANTES (regulated on activation,

normal T cell-expressed and secreted) and the cytokines interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) by 44, 71, 90, and 88%, respectively, when compared to that of cells pretreated with non-CP-SOCS1 (Fig. 6A). IP-10 and RANTES are encoded by genes that contain the IFN- γ activation sequence (GAS) promoter element (2), whereas IL-6 and G-CSF are increased in abundance in IFN- γ -stimulated cells deficient in SOCS1 relative to wild-type (WT) cells (36,37). No substantial induction of these cytokines or chemokines was observed when cells were treated with protein alone, indicating that the response was driven by IFN- γ .

Engineering of recombinant CP-SOCS1 in HEK 293 cells

Due to the persistent presence of residual LPS in *E. coli*-produced proteins (~1 ng LPS per μ g protein), we devised a strategy to produce CP-SOCS1 and non-CP-SOCS1 proteins in human embryonic kidney (HEK) 293-6E cells followed by their purification by metal-affinity fast protein liquid chromatography (FPLC) (Fig. 7A). The yield of mammalian SOCS1 proteins from HEK cells was substantially lower than that from *E. coli*; however, the recombinant proteins produced in mammalian cells had the virtue of having undetectable LPS, as determined by the *Limulus* assay. Intracellular delivery of mammalian CP-SOCS1 to AMJ2.C8 macrophages followed by their stimulation with IFN- γ attenuated the expression of chemokines [IP-10, RANTES, and macrophage inflammatory protein 1 β (MIP-1 β)] and cytokines (IL-6 and G-CSF) compared to control cells treated with non-CP-SOCS1 (Fig. 7B). Thus, these results obtained with LPS-free recombinant CP-SOCS1 and non-CP-SOCS1 proteins expressed in HEK 293-6E cells validated our results with *E. coli*-produced proteins. As before, treatment of macrophages with HEK 293-6E-produced CP-SOCS1 in the absence of IFN- γ did not have a measurable effect on the production of chemokines and cytokines. Moreover, to exclude the possibility that the attached MTM tag was responsible for the decreased production of chemokines and cytokines, we transfected HEK 293T cells with plasmids encoding non-CP-SOCS1 and CP-SOCS1 and measured the production of chemokines and cytokines in these cells after treatment with IFN- γ . We observed a substantial reduction in the production of IP-10, RANTES, and MIG (monokine induced by IFN- γ) in both non-CP-SOCS1- and CP-SOCS1-transfected cells in comparison to that of cells transfected with the vector control (fig. S4). These results indicated that the MTM tag was not responsible for CP-SOCS1-mediated suppression of the production of chemokines and cytokines. Thus, attenuation of IFN- γ -induced production of proinflammatory chemokines and cytokines depended on MTM-mediated delivery of its functionally active cargo, CP-SOCS1.

Intracellular delivery of CP-SOCS1 mutants to conduct structure-function analysis

Previous studies have indicated that the N-terminal KIR (kinase inhibitory region) and SH2 domains of SOCS1 are necessary for inhibition of JAK2-STAT1 signaling in vitro (11), whereas the SOCS box is less essential for inhibition of cytokine production in vivo (38). Moreover, the recently identified PEST domain in SOCS3 and SOCS1 (39) may contribute to their intracellular instability. Therefore, we performed mutational analysis of CP-SOCS1 to establish whether deletion of these two domains, the PEST motif and the SOCS box, would change the inhibitory activity of CP-SOCS1 upon its intracellular delivery. This analysis could identify truncated versions of CP-SOCS1 of increased stability that would be sufficient to inhibit the phosphorylation of STAT1 and the production of proinflammatory cytokines and chemokines. We constructed an N-terminally truncated form of CP-SOCS1 (CP-SOCS1 Δ PEST) by deleting amino acid residues 1 to 50 (Fig. 1), a region that harbors the PEST motif. We also engineered a double mutant (CP-SOCS1 Δ PEST.SB) lacking both the N-terminal PEST region and the amino acid residues 168 to 212, which consists of the C-terminal SOCS box (SB) domain. Pretreatment of AMJ2.C8 macrophages for 1 hour with either of these cell-penetrating mutants of CP-SOCS1 showed their preserved ability to suppress production of IL-6 when compared with that of full-length CP-SOCS1 (Fig. 6B). Thus, intracellular

delivery of cell-penetrating mutants of SOCS1 suggested that the presence of the KIR and SH2 domains was sufficient to preserve the inhibitory activity of CP-SOCS1, whereas deletion of the PEST domain or the PEST domain and the SOCS box did not impair the inhibitory activity of mutated CP-SOCS1 (Fig. 6B). These mutagenesis studies are consistent with *in vivo* studies of the SOCS1 transgene that does not contain the region encoding the SOCS box, which indicated that this mutant protein is capable of replacing functionally active full-length SOCS1 in terms of its anti-inflammatory activity (38).

Discussion

We showed that intracellular delivery of recombinant CP-SOCS1 produced either in bacterial or in mammalian cells inhibited the IFN- γ -evoked signal transduction required for the expression of genes encoding proinflammatory cytokines and chemokines in cultured macrophages. Our analysis of the mechanism of CP-SOCS1-induced attenuation of IFN- γ signaling documented its intracellular targeting of JAK2 and STAT1. Moreover, intracellular delivery of CP-SOCS1 mutant proteins that lacked the PEST and SOCS box domains suggested the central role of the KIR and SH2 domains in the attenuation of proinflammatory signaling in response to IFN- γ . Thus, our studies show the feasibility of suppressing proinflammatory signaling by the intracellular delivery of SOCS1, a key physiologic inhibitor of the IFN- γ signaling pathway. By establishing that the physiologic function of CP-SOCS1 is similar to that of endogenous SOCS1, we offer a platform for the facile study of the intracellular functions of SOCS1 because of the faster delivery, controlled input, and limited duration of CP-SOCS1 as contrasted with forced expression of the SOCS1 transgene. Moreover, by studying the mechanism of action of CP-SOCS1, we provide a starting point for the development of new therapeutics for inflammation-mediated acute syndromes, such as sepsis, the leading cause of morbidity and mortality in critical care medicine (40).

We present evidence that engineered CP-SOCS1, but not a non-CP-SOCS1 control, was able to enter cells and was resistant to digestion by proteinase K. These results are consistent with previous work from our group indicating that attachment of the MTM to protein or peptide cargo enables its intracellular delivery (32,33,41–43).

Consistent with the mechanism of action of endogenous SOCS1, we showed that CP-SOCS1 targeted the IFN- γ signaling pathway in AMJ2.C8 macrophages. It is noteworthy that the results from our immunoprecipitation experiments were not due to the induced production of endogenous SOCS1 (23.7 kD). We only observed an immunoreactive band consistent with the size of recombinant CP-SOCS1, which has a molecular mass of 27 kD, because of the added MTM and 6 $\times$ histidine tag. Moreover, the time frame over which endogenous SOCS1 protein is usually detected in response to IFN- γ stimulation is usually between 2 and 3 hours (44). Our assays were performed with IFN- γ stimulation for 5 to 10 min. Thus, our application of CP-SOCS1 for mechanistic analysis of its intracellular targets recapitulates the known action of endogenous SOCS1.

Inhibition of the phosphorylation of STAT1 by SOCS1 is due to the ability of SOCS1 to bind to the phosphorylated tyrosine residue in the activation loop of JAK2 through its central SH2 domain and the N-terminal KIR domain (45,46). Intracellular delivery of CP-SOCS1 attenuated IFN- γ -induced phosphorylation of STAT1 and the production of proinflammatory chemokines and cytokines in primary and established macrophage cell lines (Figs. 5 and 6). The extent of inhibition of the phosphorylation of STAT1 by CP-SOCS1 was dependent on its concentration. Thus, under steady-state conditions, CP-SOCS1 was effective even at low concentrations (<2.0 μ M).

Intracellular delivery of CP-SOCS1 depended on the MTM, which did not influence the intrinsic inhibitory function of CP-SOCS1. As attested by experiments involving the transfection of HEK 293F or HEK 293T cells with the CP-SOCS1 and non-CP-SOCS1 constructs, SOCS1 proteins containing or lacking the MTM equally inhibited IFN- γ -induced phosphorylation of STAT1 and the production of chemokines and cytokines (figs. S2 and S4). Thus, our data are consistent with previous reports in which ectopic expression of SOCS1 was used to inhibit phosphorylation of STAT1 and production of cytokines (7–9, 17, 36, 37, 47, 48). However, our results were accomplished by way of facile intracellular delivery of recombinant CP-SOCS1, which indicates the potential of our approach for restoring the homeostatic balance between proinflammatory stimuli and anti-inflammatory regulators, as well as its therapeutic applicability.

Until now, the production of recombinant cell-penetrating protein therapeutics was based on bacterial expression systems (32). Intractable contamination of recombinant proteins with LPS inherently present in *E. coli* prompted us to embark on designing a system to express CP proteins in a mammalian system. We succeeded in producing CP-SOCS1 and non-CP-SOCS1 in HEK 293-6E cells. Although these recombinant proteins were not as abundantly produced in these cells as they were in the bacterial expression system, they were free of LPS and displayed MTM-dependent inhibitory effects on the IFN- γ -induced production of proinflammatory chemokines and cytokines comparable to those of *E. coli*-produced proteins (Figs. 6 and 7). We showed that CP-SOCS1 inhibited the IFN- γ -dependent production of IP-10 and RANTES, which are encoded by genes that contain the GAS promoter element (2). In addition, CP-SOCS1 also inhibited the production of IL-6, G-CSF, and MIP-1 β , which are increased in abundance in IFN- γ -stimulated SOCS1-deficient cells compared to that in WT cells (36,37,47,48). The production of LPS-free recombinant CP-SOCS1 in the mammalian cell system points to the feasibility of testing this protein in animal models of inflammation, which is currently under way in our laboratory. It is noteworthy that SOCS1 proteins taken out of their intracellular milieu require protein stabilizers, such as L-arginine, a powerful suppressor of protein aggregation, to maintain protein solubility. Fortunately, CP-SOCS1 expressed in our mammalian cell system displayed increased protein solubility; nonetheless, addition of L-arginine was required, albeit at a reduced concentration compared to that required for CP-SOCS1 produced in bacteria. The technological challenges to producing recombinant SOCS1 proteins for intracellular delivery need to be overcome because of their potential use in treating multiple inflammatory disorders mediated by the uncontrolled production of proinflammatory chemokines and cytokines.

The full mechanism by which CP-SOCS1 acts likely extends beyond its inhibition of the JAK-STAT pathway. Previous reports have highlighted an important role for SOCS1 in quenching LPS signaling (26–30,49). We postulate that CP-SOCS1 use might even extend beyond the inhibition of the JAK-STAT and TLR4 pathways. Work is currently under way in which we are combining our innovative approach of intracellular protein delivery with mass spectrometry to identify potentially new interacting partners for SOCS1.

SOCS1 contains multiple domains that perform distinct roles. It inhibits the activity of JAK through its N-terminal KIR domain, a domain that is also present in SOCS3 but not in the other known members of the SOCS family (12,46,50). The centrally located SH2 domain in SOCS1 (and SOCS3) binds to phosphorylated tyrosine residues in JAK proteins and cytokine receptors. Finally, the C-terminal SOCS box serves as an E3 ubiquitin ligase that targets signaling proteins for proteasomal destruction. The latter domains, as well as the N-terminal PEST domain, contribute to the rapid turnover of SOCS proteins (39). We performed mutagenesis analysis of recombinant CP-SOCS1 proteins to determine whether loss of the N-terminal PEST domain alone or with the SOCS box domains influenced the inhibitory activity of truncated SOCS1. We showed that loss of the PEST domain did not affect the inhibitory potency of CP-SOCS1,

whereas loss of both PEST and SOCS box domains resulted in a mutant CP-SOCS1 (CP-SOCS1 Δ PEST.SB) that displayed greater activity than the full-length protein (Fig. 6B). The increased activity of CP-SOCS1 Δ PEST.SB might have been due to the loss of the PEST domain, which is responsible for increased protein turnover (39), thus leading to the increased intracellular stability of CP-SOCS1 Δ PEST.SB compared to that of full-length CP-SOCS1. Alternatively, the increased activity of CP-SOCS1 Δ PEST.SB might have been due to its smaller size compared to that of the full-length protein, which may facilitate more efficient transportation across the cell membrane than that of the full-length protein. The activities of truncated CP-SOCS1 proteins were consistent with the results of Nicholson *et al.*, which revealed that loss of these domains does not alter the activity of SOCS1 in transfected cells in vitro (11).

In conclusion, intracellular delivery of engineered, recombinant CP-SOCS1 enabled its interaction with the IFN- γ signaling pathway to attenuate the IFN- γ -induced phosphorylation of STAT1 and the production of proinflammatory cytokines and chemokines. CP-SOCS1 recapitulated the functions of endogenous SOCS1 in both transformed and primary macrophages. The development of recombinant CP-SOCS1 establishes the proof of concept of its potential utility as a therapy for inflammatory disorders triggered by acute or chronic proinflammatory cues, such as IFN- γ and LPS, which are difficult to control by currently available measures. Our work also suggests that controlled intracellular protein delivery, as a facile alternative to gene delivery, could be expanded through custom designing of recombinant CP proteins of interest to target other signaling pathways that are regulated by intracellular physiologic inhibitors.

Materials and Methods

Cell culture

The murine alveolar macrophage cell line AMJ2.C8 was obtained from the American Type Culture Collection (Manassas, VA; TIB-71) and cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc., Manassas, VA) supplemented with 5% fetal bovine serum (FBS), 10 mM Hepes, penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in 5% CO₂ in humid air. Cell viability was >80% before use in all experiments. HEK 293T cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) at 37°C in 5% CO₂ in humid air. HEK 293F cells were maintained in FreeStyle 293 medium supplemented with G418 (25 μ g/ml; Invitrogen, Carlsbad, CA) at 37°C in 8% CO₂ in humid air. HEK 293-6E cells stably expressing Epstein-Barr virus (EBV) nuclear antigen I were provided by Y. Durocher (National Research Council, Canada) and maintained in FreeStyle 293 protein expression medium supplemented with G418 (25 μ g/ml) at 37°C in 5% CO₂ in humid air.

Isolation and culture of BMDMs

For each preparation, bone marrow from C3H/HeJ mice was prepared by flushing mouse femurs and tibias with ice-cold DMEM supplemented with L-glutamine. Bone marrow cells were pooled, passed through a 25 5/8-gauge needle, and filtered through a 70- μ m cell strainer. Pooled cells (1×10^6 cells/ml) were suspended in DMEM supplemented with 10% FBS, 10 mM Hepes, penicillin (100 U/ml), streptomycin (100 μ g/ml), and 20% L929 conditioned medium followed by plating on 150-mm bacterial Petri dishes. Cells were incubated at 37°C in 5% CO₂ in humid air. Every 3 days, nonadherent cells were removed, cells were washed, and culture medium was replaced. Cells were used in experiments after 10 days of culture for up to 2 weeks after maturation. When analyzed by flow cytometry, 95% of the adherent cells were MAC3⁺, CD3⁺, and B220⁺. The viability of BMDMs was >80% before use in all experiments.

Preparation of plasmids encoding cell-penetrating SOCS1 for the production of recombinant proteins in *E. coli*

Full-length SOCS1 murine complementary DNA (cDNA) was provided by M. Shong (Chungnam National University, Korea). Polymerase chain reaction (PCR) primers encoding an MTM composed of 12 amino acid residues from a signal sequence hydrophobic region of FGF4 (33) and an Nde I site sequence at the 5' or 3' ends of SOCS1 were engineered (Integrated DNA Technologies, Coralville, IA) and used to amplify the sequence of *Socs1*. PCR products were gel-purified (Qiagen, Valencia, CA) and cloned into pCR-TOPO-2.1 according to the manufacturer's specifications and were used to transform chemically competent *E. coli* JM109 cells (Invitrogen). The 5' or 3' MTM-containing *Socs1* DNA was subsequently cloned into pET28a (EMD Chemicals, Inc., Darmstadt, Germany) and propagated in *E. coli* DH5 α (Invitrogen). The pET28a constructs containing MTM at the 5' or the 3' end of the *Socs1* sequence were transferred to *E. coli* BL21 expression vectors (Stratagene, La Jolla, CA) for determination of the abundance of SOCS1 proteins after induction with isopropyl β -D-1-thiogalactopyranoside (IPTG). *Socs1* DNA without the MTM was constructed as a control. The truncated forms of SOCS1, lacking the PEST motif and SOCS box, were constructed by PCR mutagenesis and produced in BL21 expression strains of *E. coli*.

Preparation of plasmids encoding cell-penetrating SOCS1 plasmids for the production of recombinant proteins in human cells

DNAs encoding non-CP-SOCS1 and CP-SOCS1 were subcloned into the mammalian expression vector pTT5, which were then used to transfect HEK 293-6E cells. PCR primers were constructed that encompassed a Kozak translation initiation sequence with an ATG initiation codon in front of a 6 $\times$ histidine tag and the MTM sequence. Primers contained Eco RI and Bam HI restriction site sequences to facilitate subcloning into the mammalian expression vector pTT5, which harbors the EBVoriP in the vector backbone. HEK 293-6E cells produce substantially more protein when the EBVoriP is present in the vector backbone (51, 52). Non-CP-SOCS1 was constructed similarly except for lacking the MTM sequence. DNA sequencing was performed by the Vanderbilt University DNA Sequencing Core Facility.

Production, purification, and reconstitution of recombinant SOCS1 proteins

The production of recombinant SOCS1 proteins in *E. coli* BL21 cells was induced with 0.1 to 0.5 mM IPTG and proteins were expressed as insoluble IBs. IBs were purified with the Bugbuster Protein Extraction Reagent (EMD Chemicals, Inc., Darmstadt, Germany) according to the manufacturer's protocol. Alternatively, IBs were prepared with a protocol adapted in our laboratory. Briefly, pelleted bacteria were suspended in IB buffer [20 mM tris-HCl (pH 7.5), 10 mM EDTA, 1% Triton X-100, and 0.3 M NaCl] followed by the addition of lysozyme (1.0 mg/ml) and sonication. IBs were purified by repeated centrifugation and sonication, passed through a 0.45- μ m syringe filter, and solubilized in solubilization buffer A [6 M guanidine hydrochloride (GuHCl), 100 mM NaH₂PO₄, and 10 mM tris-HCl (pH 8.0)] followed by gravity nickel-nitrilotriacetic (Qiagen) liquid chromatography. *E. coli*-derived proteins used for cytokine experiments were purified with histidine affinity columns by FPLC (AKTA Purifier, GE Healthcare, Piscataway, NJ). Briefly, proteins were bound to histidine columns in buffer A, washed extensively with buffer B [6 M GuHCl, 100 mM NaH₂PO₄, and 10 mM tris-HCl (pH 6.0)] and eluted with buffer C [6 M GuHCl, 100 mM NaH₂PO₄, and 10 mM tris-HCl (pH 4.0)]. For recombinant proteins produced in HEK 293-6E cells, pTT5 vectors containing either non-CP SOCS1 or CP-SOCS1 DNA were propagated in *E. coli* DH5 α followed by plasmid purification by cesium chloride gradient (53). Transient transfection of HEK 293-6E cells with pTT5 vectors was performed by complexing DNA with linear polyethyleneimine (PEI) (Polysciences, Warrington, CA) from a stock solution of 1 mg/ml. Briefly, DNA (1 μ g) and PEI (2 μ g) per 10⁶ cells (total $\sim$ 10⁸ cells used per transfection) were suspended in Opti-MEM

I (Invitrogen), prewarmed to 37°C, and allowed to incubate for 30 min at room temperature before being added to cells. Protein expression was allowed to proceed for 72 hours, with shaking at 125 rpm, in tissue culture flasks at 37°C in 5% CO₂ in humid air. Cells were harvested by centrifugation and suspended in buffer A, passed through a 0.2-μm filter, and purified by FPLC with a dual-step histidine purification method. Briefly, HEK-produced SOCS1 proteins were initially purified with a HisTrap FF Crude column (GE Healthcare, Piscataway, NJ) as described above, except that elution was performed under a 50-ml pH gradient from pH 6.0 to pH 4.0 after extensive washing with buffer B. Fractions containing SOCS1 proteins with a minimal number of contaminating proteins were pooled and purified again over a HisTrap HP column under similar conditions as for the crude column. With this method, the purity of SOCS1 proteins was consistently greater than 90% as quantified by the Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE). Refolding buffer conditions for each protein were established with a matrix-assisted protein refolding kit (Pierce Biotechnology, Rockford, IL). Proteins (at 100 μg/ml) were dialyzed against refolding buffer consisting of 50 mM tris-HCl, 150 mM NaCl, 0.8 mM KCl, 1.0 mM EDTA, 0.55 M GuHCl, 0.1 M NDSB 201, 0.44 M L-arginine, and 1 mM oxidized and reduced glutathione (pH 8.0) overnight at 4°C. *E. coli*-produced proteins were then exhaustively dialyzed against postrefolding buffer consisting of DMEM supplemented with 0.3 M L-arginine, 2.5 mM polyethylene glycol (PEG) 3350, and 1% penicillin and streptomycin. HEK 293-6E-produced protein was dialyzed against DMEM supplemented with 100 mM L-arginine and 1% penicillin and streptomycin. The presence of L-arginine in the postrefolding buffers was required to maintain protein stability, whereas PEG 3350 was used to minimize protein precipitation after a single freeze-thaw cycle. After dialysis, *E. coli*-produced protein solutions were passed through a 0.45-μm syringe filter and concentrated by Millipore Ultrafiltration Devices (Millipore, Billerica, MA). Concentrated proteins were used immediately for experiments, whereas nonconcentrated proteins were stored at -80°C. Any contaminating LPS in recombinant proteins was analyzed by the *Limulus* assay (Pyrosate, East Falmouth, MA) and averaged about 1.0 ng per microgram of *E. coli*-produced protein but was not detectable for proteins produced in HEK 293-6E cells.

Immunoprecipitations and Western blotting analysis

To determine whether cell-penetrating proteins could cross the cell membrane, BMDMs from C3H/HeJ mice or AMJ2.C8 macrophages were treated with equimolar concentrations of non-CP-SOCS1 (0.75 mg) and CP-SOCS1 (0.78 mg) or diluent alone for 1 hour at 37°C. Pelleted cells were washed with ice-cold DMEM containing 150 mM L-arginine (DMEM+LA) and treated with proteinase K (5 μg/ml) for 10 min at 37°C to remove proteins attached to the cell surface, followed by two additional washes in ice cold DMEM+LA. Pelleted cells were treated with lysis buffer [20 mM Hepes (pH 7.0), 2% NP-40, 50 mM KCl, 0.1 mM EDTA, and 2 mM MgCl₂] supplemented with protease inhibitors (Sigma-Aldrich, St. Louis, MO) followed by passage thorough a 25 5/8-gauge syringe needle. Lysates were cleared by centrifugation at 9000g for 15 min at 4°C followed by preclearing of the supernatant with protein G-Sepharose beads for 30 min at 4°C. Lysates containing non-CP-SOCS1 or CP-SOCS1 were immunoprecipitated with a monoclonal antibody specific for SOCS1 (5.0 μg, US Biological, Swampscott, MA) overnight at 4°C followed by incubation with protein G-Sepharose beads for 2 hours at 4°C. Where indicated, nonspecific immunoglobulin G1 antibodies (Zymed Laboratories, San Francisco, CA) were used as an immunoprecipitation control in CP-SOCS1-treated cells. Beads were washed three times with lysis buffer, followed by the elution of antibody complexes during incubation of beads in 2× SDS sample buffer at 100°C for 5 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred by Western blotting to nitrocellulose membranes, and analyzed with goat polyclonal antibodies against SOCS1 (Abcam Inc., Cambridge, MA). Western blots were developed with

fluorescently labeled secondary antibodies and visualized with the Odyssey Infrared Imaging System (LI-COR).

Analysis of protein complexes associated with intracellular CP-SOCS1

We used coimmunoprecipitation analyses to identify cellular proteins targeted by CP-SOCS1. Cells pulsed with non-CP-SOCS1 or CP-SOCS1 proteins were subjected to procedures identical to those described in the preceding section with the exception of the lysis buffer, which consisted of 20 mM Hepes (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol, and protease and phosphatase inhibitors (Sigma-Aldrich). Antibodies used for coimmunoprecipitations included monoclonal anti-SOCS1, anti-STAT1 (BD Transduction Laboratories, San Jose, CA), and anti-JAK2 (Chemicon Inc., Temecula, CA). Coimmunoprecipitation samples were subjected to SDS-PAGE and Western blot analysis with anti-STAT1 phosphorylated at Tyr⁷⁰¹ (BD Biosciences), SOCS1 (polyclonal) (Abcam), or JAK2 phosphorylated at Tyr¹⁰⁰⁷ and Tyr¹⁰⁰⁸ (Chemicon).

Analysis of STAT1 phosphorylation

BMDMs derived from C3H/HeJ mice or AMJ2.C8 cells were treated with different concentrations of non-CP-SOCS1 or CP-SOCS1 and analyzed for the extent of STAT1 phosphorylation. Cells (3.0×10^6 total cells) were suspended in medium containing the individual proteins for 1 hour followed by the addition of IFN- γ (10 to 30 U/ml; EMD Chemicals, Inc., Darmstadt, Germany) and LPS (100 ng/ml, Sigma-Aldrich). To analyze the time course of the function of CP-SOCS1, AMJ2.C8 cells were incubated with protein for 1 hour after which the protein was removed and the cells were suspended in SOCS1-free DMEM + 5% FBS (time 0). Cells were stimulated with IFN- γ (2 U/ml) starting at time 0 and at the subsequent time points. Analysis of the phosphorylation of STAT1 under conditions in which the SOCS1 proteins were expressed by transfection was performed in HEK 293F cells. Cells were transfected with the plasmids pTT5, pTT5-non-CP-SOCS1, or pTT5-CP-SOCS1 with 293fectin (Invitrogen) according to the manufacturer's specifications. After overnight incubation, 3×10^6 cells were analyzed for the extent of STAT1 phosphorylation after incubation with IFN- γ for 15 min at 37°C and 5% CO₂. For all experiments, total cell lysates were standardized for protein concentration by the method of Bradford, and the abundance of phosphorylated STAT1 was quantified by cytometric bead array (CBA, BD Biosciences) according to the manufacturer's protocol or by Western blotting analysis with an antibody specific for phosphorylated STAT1.

Analysis of the production of proinflammatory cytokines and chemokines

We analyzed the ability of non-CP-SOCS1 and CP-SOCS1 to inhibit IFN- γ -induced production of cytokines and chemokines in cultured BMDMs from C3H/HeJ mice and in AMJ2.C8 cells. Immediately before addition to cells, *E. coli*-produced protein solutions were diluted threefold, resulting in a final DMEM buffer supplemented with 100 mM L-arginine, 0.8 mM PEG 3350, 10% FBS, and 1% penicillin and streptomycin. Cells (4.0×10^5) were incubated with the appropriate protein (4.0 μ M) for 30 min followed by the addition of IFN- γ (100 U/ml) without removal of the SOCS1 proteins. Supernatants were sampled 24 hours after the addition of agonist and analyzed by the MILLIPLEX mouse cytokine-chemokine kit (Millipore, St. Charles, MO) according to the manufacturer's specifications. HEK 293-6E-produced proteins were concentrated after dialysis, diluted twofold with DMEM containing 10% FBS and 1% penicillin and streptomycin (resulting in a final DMEM buffer with 50 mM L-arginine) and used immediately in experiments. Cells (4.0×10^5) were incubated with the appropriate protein (~ 2.0 μ M) for 60 min followed by the addition of IFN- γ (30 or 100 U/ml) without removal of the SOCS1 proteins. Supernatants were sampled between 24 and 48 hours after the addition of agonist and analyzed as described above. For analysis of the effects of

SOCS1 proteins expressed by transfection on IFN- γ -induced production of cytokines under conditions of forced expression, HEK 293T cells were used. Cells were transfected with the plasmids pTT5, pTT5-non-CP-SOCS1, or pTT5-CP-SOCS1 by 293fectin as described earlier. After overnight incubation of 1.5×10^5 transfected cells in a 24-well plate, cells were stimulated for 24 hours with human IFN- γ (10 or 100 U/ml) in the presence of IL-1 β (0.1 ng/ml), followed by sampling of the supernatants. Supernatant fractions were analyzed by CBA with a human chemokine kit (BD Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Design of recombinant cell-penetrating SOCS1 proteins. The functional domains of SOCS1 and of the engineered recombinant cell-penetrating SOCS1 proteins are shown. Fusion of a hydrophobic membrane translocation motif (MTM), depicted in black, to either the C-terminal (COOH-MTM) or the N-terminal (N-MTM) portion of the protein enabled the recombinant proteins to penetrate the cell membrane. All proteins contain an N-terminal 6 $\times$ histidine (His) tag, depicted in white, to facilitate their purification from expression systems. aa, amino acids.

Fig. 2.

Expression and purification of engineered SOCS1 proteins. Recombinant protein expression was performed in *E. coli* BL21 cells either induced (+) or not induced (–) with IPTG. P, purified protein. The size of the purified proteins (in amino acid residues) and their yield from a 1-liter culture are indicated.

Fig. 3.

Intracellular delivery and detection of CP-SOCS1 but not non-CP-SOCS1. AMJ2.C8 cells were treated with diluent, non-CP-SOCS1, or CP-SOCS1 produced in bacterial cells for 1 hour, after which cell surface-associated proteins were removed by digestion with proteinase K. Cell lysates were prepared and incubated overnight with a monoclonal antibody specific for SOCS1. Antibodies were immunoprecipitated (IP) with protein G, eluted, and samples were analyzed by Western blotting with a polyclonal antibody specific for SOCS1. Results are representative of at least three experiments. As an additional control, lysates from cells treated with CP-SOCS1 were immunoprecipitated with an isotype-matched monoclonal antibody specific for Myc.

Fig. 4.

CP-SOCS1 targets the IFN- γ signaling complex. AMJ2.C8 cells were treated with non-CP- or CP-SOCS1 (produced in bacterial cells) for 1 hour, after which cells were treated with IFN- γ (100 U/ml) and LPS (0.1 μ g/ml) for 10 min. Cell surface-associated proteins were removed by digestion with proteinase K, after which cells were washed. Cell lysates were prepared and incubated with a polyclonal antibody specific for JAK2 (**A**) or a monoclonal antibody specific for STAT1 (**B**). Antibodies were immunoprecipitated (IP) with protein G, eluted, resolved by SDS-PAGE, and analyzed by Western blotting (WB) with the depicted antibodies. Results are representative of three independent experiments.

Fig. 5. CP-SOCS1 inhibits IFN- γ -induced phosphorylation of STAT1 in AMJ2.C8 cells and BMDMs. LPS-hyporesponsive AMJ2.C8 cells (**A** and **B**) and BMDMs (**C** and **D**) were treated with diluent or the indicated concentrations of non-CP-SOCS1 or CP-SOCS1 (produced in bacterial cells) followed by stimulation with IFN- γ (10 U/ml) and LPS (0.1 μ g/ml). Quantification of the abundance of phosphorylated STAT1 (pSTAT1) was performed by cytometric bead array (**A** and **C**) and confirmed by analysis of Western blots with a monoclonal antibody specific for STAT1 phosphorylated at Tyr⁷⁰¹, with detection of β -actin serving as the protein-loading control (**B** and **D**). (**A** and **C**) Error bars indicate the SEM derived from three independent experiments performed in duplicate. Cells pretreated with diluent are shown in black bars, cells pretreated with non-CP-SOCS1 in red bars, and cells pretreated with CP-SOCS1 in blue bars.

Fig. 6.

CP-SOCS1 inhibits IFN- γ -induced production of proinflammatory chemokines and cytokines by AMJ2.C8 cells. AMJ2.C8 cells were treated with the appropriate recombinant proteins (A) or with truncated CP-SOCS1 proteins (B) 60 min before stimulation with IFN- γ (100 U/ml) in the continuing presence of recombinant proteins. Supernatants were sampled 24 hours after stimulation, and the quantities of IL-6, IP-10, G-CSF, and RANTES contained in the supernatants were determined by the Millipore Milliplex mouse cytokine-chemokine kit. The results are displayed as bar graphs showing the quantities of secreted proteins (picograms per milliliter), with error bars representing the SEM. Statistical analysis was performed with the Mann-Whitney *U* test comparing the quantity of cytokine or chemokine produced from cells treated with non-CP-SOCS1 with that of cells treated with CP-SOCS1. The results are representative of three independent experiments performed in quintuplicate.

Fig. 7. Recombinant CP-SOCS1 (produced in the mammalian cell system) inhibits the IFN- γ -induced production of proinflammatory cytokines and chemokines by AMJ2.C8 cells. Production of recombinant proteins was engineered in HEK 293-6E cells transiently transfected with the indicated plasmids. **(A)** Recombinant proteins were purified by metal-affinity FPLC. AMJ2.C8 cells were treated with recombinant proteins for 60 min before stimulation with IFN- γ in the continued presence of the recombinant proteins. **(B)** Supernatants were sampled 24 to 48 hours after stimulation and the quantities of cytokines and chemokines in the samples were determined by the Millipore Milliplex mouse cytokine-chemokine kit. % Inhibition refers to the extent to which CP-SOCS1 reduced the production of a given cytokine or chemokine by cells relative to that of cells treated with non-CP-SOCS1. Statistical significance analysis was performed with the paired *t* test with a one-paired *P* value. Results are representative of at least two independent experiments performed in triplicate.