Both the Suppressor of Cytokine Signaling 1 (SOCS-1) Kinase Inhibitory Region and SOCS-1 Mimetic Bind to JAK2 Autophosphorylation Site: Implications for the Development of a SOCS-1 Antagonist


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Both the Suppressor of Cytokine Signaling 1 (SOCS-1) Kinase Inhibitory Region and SOCS-1 Mimetic Bind to JAK2 Autophosphorylation Site: Implications for the Development of a SOCS-1 Antagonist


Suppressor of cytokine signaling (SOCS)-1 protein modulates signaling by IFN-γ by binding to the autophosphorylation site of JAK2 and by targeting bound JAK2 to the proteasome for degradation. We have developed a small tyrosine kinase inhibitor peptide (Tkip) that is a SOCS-1 mimetic. Tkip is compared in this study with the kinase inhibitory region (KIR) of SOCS-1 for JAK2 recognition, inhibition of kinase activity, and regulation of IFN-γ-induced biological activity. Tkip and a peptide corresponding to the KIR of SOCS-1, (53)DTHFRTFRSHSDYRRI(68) (SOCS1-KIR), both bound similarly to the autophosphorylation site of JAK2, JAK2(1001–1013). The peptides also bound to JAK2 peptide phosphorylated at Tyr1007, pJAK2(1001–1013). Dose-response competitions suggest that Tkip and SOCS1-KIR similarly recognize the autophosphorylation site of JAK2, but probably not precisely the same way. Although Tkip inhibited JAK2 autophosphorylation as well as IFN-γ-induced STAT1-α phosphorylation, SOCS1-KIR, like SOCS-1, did not inhibit JAK2 autophosphorylation but inhibited STAT1-α activation. Both Tkip and SOCS1-KIR inhibited IFN-γ activation of Raw 264.7 murine macrophages and inhibited Ag-specific splenocyte proliferation. The fact that SOCS1-KIR binds to pJAK2(1001–1013) suggests that the JAK2 peptide could function as an antagonist of SOCS-1. Thus, pJAK2(1001–1013) enhanced suboptimal IFN-γ activity, blocked SOCS-1-induced inhibition of STAT3 phosphorylation in IL-6-treated cells, enhanced IFN-γ activation site promoter activity, and enhanced Ag-specific proliferation. Furthermore, SOCS-1 competed with SOCS1-KIR for pJAK2(1001–1013). Thus, the KIR region of SOCS-1 binds directly to the autophosphorylation site of JAK2 and a peptide corresponding to this site can function as an antagonist of SOCS-1. The Journal of Immunology, 2007, 178: 5058–5068.

The JAKs are a small but indispensable enzyme family that mediate the biological effects of cytokines, hormones, and growth factors by tyrosine phosphorylation of STATs (reviewed in Refs. 1 and 2). The IFNs, including types I and type II, hormones such as growth hormone and angiotensin, and growth factors such as thrombopoietin, are all dependent on JAK tyrosine phosphorylation of appropriate STAT transcription factors for their physiological functions (3, 4).

The immediate early signal transduction events associated with IFN-γ and its receptor subunits involve the obligatory action of two tyrosine kinases, JAK1 and JAK2 (reviewed in Ref. 5). The IFN-γ receptor (IFNGR)5 system is a heterodimeric complex consisting of an α-subunit, IFNGR-1, and a β-subunit, IFNGR-2, both of which are essential for the biological activity of IFN-γ (5). JAK1 is associated with the IFNGR-1 chain, whereas JAK2 is associated with the IFNGR-2 chain. The interaction of IFN-γ, primarily with the IFNGR-1 subunit, initiates a sequence of events that results in increased binding of JAK2 to IFNGR-1. This has important consequences for subsequent critical phosphorylation events. JAK2, in the process of binding to IFNGR-1, undergoes autophosphorylation, and at the same time IFNGR-1 is phosphorylated. These events occur in concert with JAK1 function, resulting in recruitment and tyrosine phosphorylation of the IFN-γ transcription factor, STAT1α (Ref. 5; reviewed in Ref. 6).

A family of proteins called suppressors of cytokine signaling (SOCS) negatively regulates JAK/STAT signaling (7–9). These inducible proteins share domains of homology that characterize the SOCS family. SOCS proteins are also negative regulators of signaling by other cytokines, growth factors, and hormones (reviewed in Refs. 10–12). There are currently eight identified members of the SOCS family, SOCS-1 to SOCS-7 and cytokine-inducible SH2 protein. SOCS-1 is of particular interest, because it is a negative regulator of JAK2 as well as several other cytokines and hormone inhibitor peptide; SH2, Src homology 2; KIR, kinase inhibitory region; lipo, lipophilic; MuIFNγ, murine IFN-γ; GAS, IFN-γ-activated sequence; MBP, myelin basic protein; EMCV, encephalomyocarditis virus; p, phosphorylated; ESS, extended SH2 sequence; sRNA, small-interfering RNA; EAE, experimental allergic encephalomyelitis.

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receptor systems, including epidermal growth factor receptor (EGFR) (reviewed in Ref. 13).

Our laboratory recently designed a short 12-mer peptide, WLVFFVIFYFFR, which binds to the autophosphorylation site of JAK2, resulting in inhibition of its autophosphorylation as well as its phosphorylation of IFNGR subunit IFNGR-1 (14). The JAK2 tyrosine kinase inhibitor peptide (Tkip) did not bind to or inhibit tyrosine phosphorylation of vascular endothelial growth factor receptor or phosphorylation of a substrate peptide by the proto-oncogene tyrosine kinase c-Src. Tkip, as with SOCS-1, also inhibited EGFR autophosphorylation. It has been suggested that SOCS-1 specifically recognizes the autophosphorylation sequence 1001–1013 containing the phosphotyrosine residue (pY1007) in the activation loop of JAK2 and that the phosphorylation of Y1007 is required for activation (15). SOCS-1 binding blocks JAK2 tyrosine phosphorylation of substrate. Tkip recognizes both unphosphorylated and phosphorylated (pY1007) with higher affinity for pY1007. Thus, Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1. Tkip has also been shown to inhibit proliferation of prostate cancer cells, blocking JAK2 phosphorylation and activation of the oncogene STAT3 (16). Furthermore, Tkip has been shown to protect mice from experimental allergic encephalomyelitis (EAE), an animal model for multiple sclerosis, via blockage of JAK2 activation of inflammatory cytokines (17). Hence, Tkip appears to have both anti-inflammatory and antitumor properties.

It has been suggested that the binding of SOCS-1 to JAK2 requires the SOCS-1 Src homology 2 (SH2) domain and that the kinase inhibitory region (KIR), while not required for the binding, is essential for the inhibitory action of SOCS-1 (15). We show here that a peptide corresponding to SOCS-1 KIR, SOCS1-KIR peptide, specifically binds to a peptide representing the JAK2 autophosphorylation site and inhibits STAT1 activation. Furthermore, we show that SOCS1-KIR, as well as Tkip, inhibit IFN-γ-induced macrophage activation. We also present data on a novel SOCS-1 antagonist peptide, pJAK2(1001–1013), that enhances suboptimal IFN-γ-induced antiviral activity, enhances IFN-γ-activated sequences (GAS) promoter activity, inhibits SOCS-1 suppression of STAT3 activation of LNCaP prostate cancer cells, and enhances Ag-specific splenocytes proliferation.

Materials and Methods

Cell culture and virus

Cell lines, except S9 cells, were obtained from American Type Culture Collection. The human prostate cancer cells, LNCaP, and the murine macrophage cells, Raw 264.7, were maintained in RPMI 1640 medium (SAFC Biosciences) supplemented with 10% FBS (HyClone), 100 U/ml penicillin and 100 U/ml streptomycin (complete medium). Murine fibroblast cells, L929, were maintained in DMEM (SAFC Biosciences) medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. The murine monococytes, U937, were maintained in RPMI 1640 complete medium supplemented with 10 mM HEPES (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). All these cell types were cultured at 37°C and 5% CO₂ atmosphere. Sf9 insect cells were purchased from Invitrogen Life Technologies and were maintained as adherent cultures in TNM-FH medium or as suspension cultures in SF-900 II SFM medium (Invitrogen Life Technologies) at 27°C.

Peptides

The peptides used in this study are listed in Table I and were synthesized in our laboratory on an Applied Biosystems 9050 automated peptide synthesizer using conventional fluorenlymethoxy carbonyl chemistry as described previously (18). A lipophilic (lipo) group (palmitoyl-lysine) was added to the N terminus of peptides to facilitate entry into cells as a last step using a semiautomated protocol. Peptides were characterized by mass spectrometry and purified by HPLC. Peptides were dissolved in water or in DMSO (Sigma-Aldrich).

Binding assays

Binding assays were performed as previously described (14) with minor modifications. Tkip, SOCS1-KIR, and control peptides were bound to 96-well plates, in binding buffer (0.1 M carbonate-bicarbonate (pH 9.6)), at a final concentration of 3 μg/well. Wells were then washed in wash buffer (PBS containing 0.9% NaCl and 0.05% Tween 20), blocked with 2% gelatin and 0.05% Tween 20 in PBS for 1 h at room temperature, washed three times with wash buffer, and incubated with various concentrations of biotinylated JAK2(1001–1013) or biotinylated pJAK2(1001–1013) for 1 h at room temperature in blocking buffer. Following incubation, wells were washed five times to remove unbound biotinylated peptide. Bound biotinylated peptides were detected by incubation with 1/500 dilution of neutravidin-biotin-binding protein conjugated with HRP (Molecular Probes) in blocking buffer for 1 h at room temperature. Wells were then washed five times with wash buffer and incubated with a solution of o-phenylenediamine in stable peroxidase buffer (Pierce) and color was allowed to develop.

Peptide competition assays were conducted as described above except following peptide immobilization, washing and blocking, biotinylated JAK2(1001–1013) or biotinylated pJAK2(1001–1013), which had been preincubated (for 1 h at room temperature) with various concentrations of soluble peptide competitors (Tkip, SOCS1-KIR, or control peptide), was added. Detection of bound biotinylated peptide was conducted as described above. Data obtained from binding assays were plotted using GraphPad Prism 4.0 software.

In vitro kinase assays

Autophosphorylation activity of JAK2 was measured in a reaction mixture containing GST-JAK2 kinase fusion protein (Cell Signaling Technology), ATP (20 μM, Cell Signaling Technology), and the appropriate peptide in kinase buffer (10 mM HEPES (pH 7.4), 50 mM sodium chloride, 0.1 mM sodium orthovanadate, 5 mM magnesium chloride, and 5 mM manganese.
chloride). It had been determined previously that soluble IFNγR-1 enhanced JAK2 activity (14). Hence, IFNγR1 at 2 μg/reaction was added followed by incubation at 25°C for 30 min with intermittent agitation. The reactions were terminated by addition of appropriate volume of 6X SDS-PAGE loading buffer (0.5 M Tris-HCl (pH 6.8), 36% glycerol, 10% SDS, 9.3% DTT, and 0.012% bromphenol blue) and incubation at 95°C for 5 min. The proteins were separated on a 12% SDS-polyacrylamide gel (Bio-Rad), transferred onto a nitrocellulose membrane (Amersham Biosciences), and probed with anti-JAK2 Ab (Santa Cruz Biotechnology). Membranes were stripped and reprobed with anti-JAK2 Ab (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL protein detection reagents (Amersham Biosciences).

**Immunoblot analysis of STAT1α**

U937 murine fibroblast cells were plated on 6-well plates at a cell density of 1 × 10⁶ cells/well and after an overnight incubation at 37°C and 5% CO₂, the cells were cultured in complete medium containing varying concentrations of lipo Tkip, lipo SOCS1-KIR, or lipo control peptide for 18 h at 37°C in a 5% CO₂ incubator. To activate the JAK/STAT-signaling pathway, the cells were incubated in the presence or absence of 1000 U/ml IFN-γ (PBL Biomedical Laboratories) for an additional 30 min. The medium was aspirated out and the cells washed twice with cold PBS to remove medium and cell debris. Cell lysates were prepared by adding 250 μl of cold lysis buffer (50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 50 mM NaF, 5 mM EDTA, and 0.1% Nonidet P-40) containing protease inhibitor mixture (Amersham Biosciences) and phosphatase inhibitors (Sigma-Aldrich, Inc.). The cell lysates were then subjected to complete cell lysis. Lysates were then centrifuged to remove cellular debris and the supernatant was transferred to fresh microcentrifuge tubes. Protein concentrations were determined and protein lysates were resolved by SDS-PAGE on a 12% polyacrylamide gel (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Amersham Biosciences), nonspecific sites were blocked in blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in TBS), and washed in wash buffer (0.1% Tween 20 in TBS). To detect pSTAT1α, membranes were incubated with pY701-STAT1α Ab (1/400 dilution; Santa Cruz Biotechnology) in blocking buffer for 2 h at room temperature. After three washes in wash buffer, the membranes were incubated in HRP-conjugated goat anti-rabbit IgG secondary Ab (1/2000 dilution; Santa Cruz Biotechnology) in blocking buffer for 1 h at room temperature. Following three washes in wash buffer, membranes were incubated with ECL detection reagents (Amersham Biosciences) and exposed to photographic film (Amersham Biosciences) to visualize protein bands.

**Macrophage activity**

Murine macrophage cells, Raw 264.7, were seeded on 24-well plates at a concentration of 3 × 10⁵ cells/well (300 μl/well) and allowed to adhere. Varying concentrations of lipo peptides, Tkip, SOCS1-KIR, or murine IFNγR1 (Upstate Biotechnology), were then added to the wells and the cells incubated for 2 h at 37°C in a 5% CO₂ incubator. Varying concentrations of IFN-γ were then added and the cells were incubated for an additional 72 h at 37°C in a 5% CO₂ incubator, after which supernatants were transferred to fresh tubes and assayed for nitrite levels as a measure of NO production using Griess reagent, according to the manufacturer’s instructions (Alexis Biosciences). To test for synergy between Tkip and SOCS1-KIR, the cells were incubated in the presence of IFN-γ and varying concentrations of peptides as described above and also in the presence of both lipo Tkip and lipo SOCS1-KIR or lipo Tkip and lipo MuIFNγ (95–106) and were collected after 48 h and tested for NO production as described above.

**Ag-specific proliferation assay**

SJL/J mice were immunized with bovine myelin basic protein (MBP) as previously described (17), and spleens were extracted and homogenized into a single-cell suspension. Splenocytes (1 × 10⁵ cells/well) were incubated with medium, MBP (50 μg/ml), lipo Tkip, lipo SOCS1-KIR, or lipo MuIFNγ (253–287) for 48 h at 37°C in 5% CO₂. The cultures were pulsed with [3H]thymidine (1.0 μCi/well; Amersham Biosciences) 18 h before harvesting into filter paper discs using a cell harvester. Cell-associated radioactivity was quantified using a beta scintillation counter, and data are reported as counts per minute.

**Antiviral assays for SOCS-1 antagonist function**

Antiviral activity was determined using a standard viral cytopathic effect assay as described previously with minor modifications (19). Briefly, human fibroblast WISH cells at 70–80% confluency were incubated in medium alone or 0.4 U/ml IFN-γ (PBL Biomedical Laboratories) or both 0.4 U/ml IFN-γ and lipo pJAK2(1001–1013) or lipo JAK2(1001–1013) for 22 h in maintenance medium. Following incubation, WISH cells were washed once with maintenance medium and infected with encephalomyocarditis virus (EMCV) at 0.1 PFU/WISH cell for 1 h at 37°C. WISH cells were then washed once to remove unbound viral particles and incubated in fresh maintenance medium for an additional 24 h at 37°C in a 5% CO₂ incubator. Plates were subsequently blotted dry and stained with 0.1% crystal violet solution for 5 min. Unbound crystal violet was aspirated and the plates were thoroughly rinsed with deionized water, blotted, and air-dried. Virus plaques were counted using a dissecting microscope and percentages of cell survival were determined by comparing experimental treatment groups with the virus-only control group.

**Transfection of mammalian cells with SOCS-1 DNA**

Transfections were conducted to introduce SOCS-1 DNA into mammalian cells and test the ability of pJAK2 peptide to reverse SOCS-1 inhibition of STAT3 phosphorylation. Human prostate cancer cells, LNCaP, were plated in a 6-well plate and allowed to grow to 60% confluency. SOCS-1 plasmid DNA (1.6 μg/well) (pEF-FLAG-I/mSOCS1), a gift from Dr. D. Hilton (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia; Ref. 7) or an empty vector, was transfected into the LNCaP cells using LipofectAMINE (Invitrogen Life Technologies) and incubated for 4 h, after which the transfection medium was aspirated and fresh complete medium (Eagle’s MEM supplemented with 10% FBS and 100 U/ml streptomycin and 100 U/ml penicillin) was added and the cells were incubated for 72 h at 37°C. The complete medium was then aspirated, and fresh medium containing lipo pJAK2(1001–1013) (20 μM) or control peptide (lipo MuIFNγ (95–125)) was added to the transfected cells, and the JAK/STAT-signaling pathway was activated by adding IL-6 (50 ng/ml). Cells were incubated for 30 min before harvesting. Cell extracts were resolved by SDS-PAGE on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane (Amersham Biosciences), and probed with phosphorylated pY701 STAT3 Ab (Santa Cruz Biotechnology). The membranes were stripped and reprobed with unphosphorylated STAT3 Ab (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL detection reagents (Amersham Biosciences).

**For immunoprecipitations, LNCaP cells growing on 60-mm plates and at 50% confluency were transfected with either SOCS-1 plasmid DNA (8 μg/plate) or empty vector (8 μg/plate) in LipofectAMINE and incubated for 4 h, after which 5 ml of complete medium was added and cells were incubated for 72 h. The cells extracts were prepared as described for Western blot analysis and incubated with 2 μg/ml anti-FLAG Ab (Sigma-Aldrich) for 2 h at 4°C followed by incubation with Protein G PLUS-agarose beads (Santa Cruz Biotechnology) for an additional 2 h at 4°C. Following centrifugation to pellet protein G immune complexes, supernatants were discarded, immune complexes were washed three times with lysis buffer and once with PBS. The immune complexes were heated (95°C/25 min) in 50 μl of 1 × SDS sample buffer, resolved on a 12% polyacrylamide gel, transferred onto nitrocellulose, and immunoblotted with anti-SOCS-1 Ab (Santa Cruz Biotechnology). Detection of proteins was accomplished using ECL detection reagents (Amersham Biosciences).**

**IFN-γ-activated sequence (GAS) promoter activity**

A plasmid, pGAS-Luc, that contains the promoter for GAS linked to the firefly luciferase gene was obtained from Stratagene. A constitutively expressed myelin basic protein promoter-driven Renilla luciferase gene plasmid (pRl-TK) (Promega) was used as internal control in reporter gene transfections. WISH cells (1 × 10⁶ cells/well) were seeded in a 12-well plate and incubated overnight at 37°C, following which 3 μg of GAS promoter-driven firefly luciferase expressing plasmid DNA and 10 ng of pRL-TK were cotransfected, into the WISH cells, using Fugene 6 (Roche Diagnostics). Two days later, the cell lysates were used to assay for firefly luciferase and Renilla luciferase, using a dual luciferase assay kit (Promega). Luciferase activity, in relative luciferase units, was calculated by dividing firefly luciferase activity by Renilla luciferase activity in each sample.
Expression of SOCS-1 protein in Sf9 insect cells

Murine SOCS-1 DNA was PCR amplified from the SOCS-1 expression vector, pEF-FLAG-I/mSOCS-1, a gift from Dr. D. Hilton. The PCR products were purified and cloned into the pBlueBac4.5/V5-His TOPO TA expression vector (Invitrogen Life Technologies) and the recombinant vector was used to transform TOP10 chemically competent cells according to the manufacturer’s instructions (Invitrogen Life Technologies). Positive clones were identified by PCR, plasmid DNA was isolated, and the presence of SOCS-1 DNA was confirmed by restriction enzyme digestion and sequencing. The recombinant vector, pBlueBac4.5/V5-His-V5 TOPO, carrying the murine SOCS-1 gene (pBlueBac4.5-His/muSOCS-1), and Bac-N-Blue vector were then cotransfected into Sf9 insect cells (Invitrogen Life Technologies) according to the manufacturer’s instructions. Positive viral constructs, identified by color selection and PCR, were propagated in Sf9 cells to give high titer virus, which were then used to infect 250 ml of Sf9 cells growing in suspension culture. Four days after infection, the Sf9 cells were harvested and protein was extracted using native conditions as described in the Invitrogen Life Technologies instruction manual. Control reactions were conducted similar to SOCS-1 expression except empty vectors were used. Cell lysates were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted with anti-SOCS-1 Ab. Lysates were also used for competition for binding assays, which were conducted as described previously.

Results

Both Tkip and SOCS1-KIR bind to the JAK2 autophosphorylation site

We first determined whether SOCS1-KIR, like the SOCS-1 mimetic Tkip, binds to the JAK2 autophosphorylation site by carrying out dose-response solid-phase binding assays with the JAK2 autophosphorylation site peptide, JAK2(1001–1013). Tkip, SOCS1-KIR, or a control peptide, MuIFN (95–106), were immobilized on 96-well microtiter plates and incubated with biotynilated JAK2(1001–1013) at various concentrations. Tkip and SOCS1-KIR, but not the control peptide, bound to the JAK2(1001–1013) peptide in a dose-dependent manner (Fig. 1A). Thus, both Tkip and SOCS1-KIR specifically bind to the JAK2 autophosphorylation site peptide. Although this is consistent with the SOCS-1 mimetic character of Tkip, it also provides direct evidence that the SOCS1-KIR region of SOCS-1 can interact directly with the JAK2 autophosphorylation site.

Because phosphorylation of Y1007 is required for high catalytic activity of JAK2, it is logical that SOCS-1 would bind with higher affinity to Y1007 pJAK2. Consistent with this, we have previously shown that Tkip binds with greater affinity to the Y1007 pJAK2(1001–1013) peptide, pJAK2(1001–1013), than to the unphosphorylated JAK2 peptide (14). Therefore, we determined whether SOCS1-KIR bound to pJAK2(1001–1013) with greater affinity than to JAK2(1001–1013). Both SOCS1-KIR and Tkip bound to pJAK2(1001–1013) with 2- to 3-fold greater affinity than to JAK2(1001–1013) as shown in Fig. 1B. Thus, SOCS1-KIR recognizes JAK2 autophosphorylation similar to Tkip, the implication of which is that Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1.

To determine whether Tkip and SOCS1-KIR bind to the same site on the JAK2 autophosphorylation site, binding competition assays were conducted. Tkip or SOCS1-KIR was immobilized on a 96-well plate and biotynilated JAK2(1001–1013) or biotynilated pJAK2(1001–1013), which had been preincubated with Tkip, SOCS1-KIR, or a control peptide, was allowed to bind to the immobilized peptides. As shown in Fig. 2A, soluble Tkip and soluble SOCS1-KIR, but not soluble control peptide, inhibited the binding of biotynilated JAK2(1001–1013) to immobilized Tkip. A similar pattern of inhibition was observed with biotynilated JAK2(1001–1013) binding to immobilized SOCS1-KIR (Fig. 2B). Homologous inhibition was slightly better for both Tkip and SOCS1-KIR, which suggests slight differences in recognition of the JAK2 autophosphorylation site.

We next determined the binding competition of Tkip and SOCS1-KIR to pJAK2(1001–1013), which was similar to competition for binding to the unphosphorylated JAK2 peptide (Fig. 2, C and D). Homologous competition was slightly better, which again suggests slight differences in recognition of the JAK2 autophosphorylation site. These data provide direct evidence that the mimetic effect of Tkip is applicable to the KIR of SOCS-1.

JAK2 kinase activity and STAT1α activation

SOCS-1 regulates JAK2 activity at least at two levels. One involves interaction with the autophosphorylation site, which affects JAK2 phosphorylation of substrates such as STAT1 and STAT3 (15). The other level involves induction of proteosomal degradation of both JAK2 and SOCS-1, requiring the SOCS box domain of SOCS-1 (20). Obviously, neither Tkip nor SOCS-KIR has a SOCS box, so we compared the two peptides for their relative ability to inhibit JAK2 autophosphorylation as well as phosphorylation of the transcription factor, STAT1. As shown in Fig. 3A, Tkip but not SOCS1-KIR inhibited JAK2 autophosphorylation.
This would suggest that the similar but slight differences in recognition of JAK2 resulted in significant differences in regulation of JAK2 autophosphorylation.

We next compared the two peptides for their relative ability to inhibit IFN-γ activation of STAT1α in murine U937 cells. In contrast to inhibition of JAK2 autophosphorylation, both Tkip and SOCS1-KIR inhibited JAK2-mediated phosphorylation of STAT1α (Fig. 3B). Thus, Tkip inhibits JAK2 autophosphorylation as well as JAK2-mediated phosphorylation of STAT1α in murine U937 cells, whereas SOCS1-KIR does not inhibit JAK2 autophosphorylation but does inhibit JAK2-mediated phosphorylation of the STAT1α transcription factor. SOCS1-KIR thus shows the same regulatory pattern as SOCS-1, in that JAK2 autophosphorylation is not inhibited, whereas STAT1α is substrate phosphorylation by activated JAK2 is inhibited. We previously showed that Tkip, as with SOCS-1, also inhibited EGFR autophosphorylation (14). Thus, we compared SOCS1-KIR with Tkip in inhibition of EGFR phosphorylation. As shown in Fig. 3C, both peptides inhibited EGFR phosphorylation with Tkip being the more effective inhibitor. SOCS1-KIR is thus similar to SOCS-1 in its kinase inhibitory function.

**FIGURE 2.** Both soluble SOCS1-KIR and soluble Tkip inhibit the binding of biotinylated JAK2(1001–1013) and biotinylated pJAK2(1001–1013) to immobilized Tkip or SOCS1-KIR. A, SOCS1-KIR and Tkip inhibit the binding of JAK2(1001–1013) to immobilized Tkip. Biotinylated JAK2(1001–1013) (500 μM), which had been preincubated with varying concentrations of soluble peptide competitors Tkip, SOCS1-KIR, or control peptide, was added in triplicate to a 96-well plate coated with Tkip, bound biotinylated JAK2(1001–1013) was detected by ELISA using a neutravidin-HRP conjugate as described in the Materials and Methods. B, SOCS1-KIR and Tkip inhibit the binding of JAK2(1001–1013) to immobilized peptide. Biotinylated JAK2(1001–1013) (500 μM), which had been preincubated with varying concentrations of soluble peptide competitors Tkip, SOCS1-KIR, or control peptide, was added in triplicate to a 96-well plate coated with SOCS1-KIR. Bound biotinylated JAK2(1001–1013) was detected by ELISA using a neutravidin-HRP conjugate as described in Materials and Methods. C, SOCS1-KIR and Tkip inhibit the binding of pJAK2(1001–1013) to immobilized Tkip. Biotinylated pJAK2(1001–1013) (500 μM) that had been preincubated with varying concentrations of soluble peptide competitors Tkip, SOCS1-KIR, or control peptide was added in triplicate to a 96-well plate coated with Tkip. Bound biotinylated pJAK2(1001–1013) was detected by ELISA. D, SOCS1-KIR and Tkip inhibit the binding of pJAK2(1001–1013) to immobilized SOCS1-KIR. Biotinylated pJAK2(1001–1013) (500 μM) that had been preincubated with varying concentrations of soluble peptide competitors Tkip, SOCS1-KIR, or control peptide, was added and tested for inhibition of binding. Bound biotinylated pJAK2(1001–1013) was detected by ELISA. For all competition for binding assays, the differences in inhibition of binding by Tkip or SOCS1-KIR, when compared with the control peptide, were statistically significant as determined using two-way ANOVA (p < 0.0001). All experiments were conducted in triplicate and the data are representative of three independent experiments.
Tkip in JAK2 autophosphorylation, STAT1

**FIGURE 3.** Differences in kinase inhibition patterns of SOCS1-KIR and Abs to pSTAT1

SDS-PAGE, transferred to nitrocellulose membrane, and examined using washed, harvested, and lysed. Whole cell extracts were resolved on 12% bation in the presence or absence of IFN-peptide MuIFNGR1 (253–287) for 18 h at 37°C. Following 30-min incu-
pretreated with medium alone, lipo Tkip, lipo SOCS1-KIR, or lipo control peptide or SOCS1-KIR, inhibited JAK2 autophosphorylation. Tkip, but not the Immuno-blotted with anti-JAK2 Ab. Tkip, but not SOCS1-KIR, inhibited IFN-

activation in U937 cells. U937 cells were seeded into 6-well plates at 1/100,000 cells/well and

The demonstration above that the KIR of SOCS-1 can bind to the 12-mer ESS. The specific role of the various residues in the protein, it has been proposed that the SH2 domain plus ESS binds to JAK2 at the activation site represented by peptide pJAK2(1001–1013), whereas KIR binds primarily to the catalytic site of JAK2 (15). Therefore, we synthesized the ESS peptide, 68ITRASALL, DACG, and compared it with SOCS1-KIR for binding to biotinylated pJAK2(1001–1013). As shown in Fig. 6, SOCS1-KIR as well as Tkip bound biotin-pJAK2(1001–1013) in a dose-response manner, while ESS failed to bind. Our SOCS1-KIR sequence, except for residues 53–55, is contained in the SOCS-1 ESS-SH2 construct (dn56) in the binding experiment of SOCS-1 (15). Thus, KIR, which is N-terminal and contiguous with ESS, probably shares overlapping functional sites with ESS. Clearly, SOCS1-KIR is preferentially recognized by JAK2 (1001–1013) compared with the 12-mer ESS. The specific role of the various residues in the SH2 domain of SOCS-1 in JAK2 and pJAK2(1001–1013) binding remains to be determined.

**SOCS-1 antagonist activity of pJAK2 autophosphorylation site peptide pJAK2(1001–1013)**

Based in part on binding experiments with truncations of SOCS-1 protein, it has been proposed that the SH2 domain plus ESS binds to JAK2 at the activation site represented by peptide pJAK2(1001–1013), whereas KIR binds primarily to the catalytic site of JAK2 (15). Therefore, we synthesized the ESS peptide, 68ITRASALL, DACG, and compared it with SOCS1-KIR for binding to biotinylated pJAK2(1001–1013). As shown in Fig. 6, SOCS1-KIR as well as Tkip bound biotin-pJAK2(1001–1013) in a dose-response manner, while ESS failed to bind. Our SOCS1-KIR sequence, except for residues 53–55, is contained in the SOCS-1 ESS-SH2 construct (dn56) in the binding experiment of SOCS-1 (15). Thus, KIR, which is N-terminal and contiguous with ESS, probably shares overlapping functional sites with ESS. Clearly, SOCS1-KIR is preferentially recognized by JAK2 (1001–1013) compared with the 12-mer ESS. The specific role of the various residues in the SH2 domain of SOCS-1 in JAK2 and pJAK2(1001–1013) binding remains to be determined.

**Twelve-residue extended SH2 sequence (ESS) peptide does not bind to pJAK2(1001–1013)**

We have previously shown that Tkip inhibits Ag-specific proliferation of mouse splenocytes in vitro (23). Specifically, Tkip inhibited proliferation of splenocytes from mice immunized with bovine MBP. In this study, we compared Tkip and SOCS1-KIR for their relative ability to inhibit proliferation of MBP-specific splenocytes in cell culture. Splenocytes (3 × 10^5 cells/well) were incubated with MBP (50 µg/ml) in the presence of lipo Tkip, lipo SOCS1-KIR, or lipo control peptide for 48 h and proliferation was assessed by testing for [3H]thymidine incorporation. As shown in Fig. 5, both Tkip and SOCS1-KIR inhibited MBP-induced proliferation of splenocytes, while the control peptide had a negligible effect on proliferation. Similar to inhibition of NO production by macrophages, Tkip was more effective than SOCS1-KIR in inhibition of MBP-induced splenocyte proliferation with 84, 88, and 97% inhibition at 1.2, 3.7, and 11 µM, respectively, compared with 61, 67, and 72% for SOCS1-KIR. Thus, both Tkip and SOCS1-KIR inhibited Ag-induced splenocyte proliferation, which is consistent with SOCS-1 protein inhibition of Ag-specific lymphocyte activity (24).

**STAT1α Ab (lower panel).** C. Both Tkip and SOCS1-KIR inhibit EGFR phosphorylation. In vitro kinase assays were conducted in which SOCS1-KIR, Tkip, or control peptide was incubated with EGF and EGFR and ATP for 30 min at 25°C. The kinase reaction mixtures were resolved on 12% SDS-PAGE, transferred onto nitrocellulose membrane, and immunoblotted with anti-phosphorylated EGFR Ab (upper panel), with the densitometry readings of band intensities shown. The membrane was stripped and re-probed with STAT1α Ab (lower panel). Both Tkip and SOCS1-KIR inhibited EGFR phosphorylation.
As shown in Fig. 7A, the antiviral activity of a suboptimal dose of IFN-γ (0.4 U/ml) was enhanced against encephalomyocarditis virus (EMCV) in WISH cells by pJAK2(1001–1013). Specifically, unphosphorylated JAK2(1001–1013) at 11 M final concentration reduced EMCV plaques relative to IFN-γ alone by 42%, whereas the same concentration of pJAK2(1001–1013) reduced plaques by 59%. This is consistent with better binding of pJAK2(1001–1013) by SOCS1-KIR as shown above and by previous studies showing that SOCS-1 is active against JAK2 phosphorylated at Tyr1007 (15). The peptide alone had no effect on EMCV, similar to medium (data not shown). Thus, pJAK2(1001–1013) boosts the activity of a suboptimal concentration of IFN-γ, possibly interfering with endogenous SOCS-1 activity.

At the level of signal transduction, we examined the effects of pJAK2(1001–1013) on activation of STAT3 transcription factor in the LNCaP prostate cancer cell line. The cells were treated with IL-6 to activate STAT3 signaling, which occurs through JAK2 kinase (16). We overexpressed the SOCS-1 gene in these cells, which reduced the level of IL-6-induced activation of phospho-STAT3 as shown in Fig. 7B. Treatment of the cells with pJAK2(1001–1013) (20 M) resulted in a 2-fold increase in activated STAT3 compared with IL-6-treated cells that were transfected with SOCS-1, as per densitometry readings. Expression of SOCS-1 proteins in LNCaP cells is shown in Fig. 7B. Thus,
pJAK2(1001–1013) has an inhibitory effect on SOCS-1 at the level of signal transduction. We next determined whether pJAK2(1001–1013) could enhance suboptimal IFN-γ-induced antiviral activity against EMCV in tissue culture. Human fibroblast WISH cells, seeded to confluence in a 24-well plate, were pretreated with human IFN-γ (0.4 U/ml) and 11 μM pJAK2(1001–1013) or 11 μM JAK2(1001–1013) and incubated for 22 h, after which cells were challenged with EMCV (200 PFU/well) for 1 h. The virus was then removed, and medium was added. Cells were incubated for at least 24 h and then stained with 0.1% crystal violet solution. Unbound crystal violet was aspirated and plates were rinsed thoroughly with deionized water, blotted dry, and air-dried. Viral plaques were counted on a dissecting microscope. B, pJAK2(1001–1013) reverses SOCS-1 inhibition of STAT3 phosphorylation. Human prostate cancer (LNCaP) cells were plated on a 6-well plate and allowed to grow to 60% confluency. SOCS-1 plasmid DNA (8 μg/well) (pEF-FLAG-I/mSOCS1), provided by Dr. D. Hilton, or empty vector was transfected into the LNCaP cells, using LipofectAMINE. Lipo pJAK2(1001–1013) (20 μM) or an equivalent volume of medium was added to the transfected cells, and the JAK/STAT-signaling pathway was activated by adding IL-6 (50 ng/ml). Cells were incubated for 30 min before harvesting. Cell extracts were resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, and examined using Abs to pSTAT3 (pY705) (upper panel), and the densitometry readings of band intensities are shown. The membranes were stripped and reprobed with unphosphorylated STAT3 Ab (middle panel), and SOCS-1 expression in LNCaP cells is shown in the lower panel. The data are representative of two independent experiments. C, pJAK2(1001–1013) enhances GAS promoter activity. WISH cells were transfected with a vector-expressing firefly luciferase, driven by a GAS promoter, along with a vector expressing Renilla luciferase as a control vector. IFN-γ (1 U/ml) and lipo pJAK2(1001–1013) at 5 or 1 μM final concentration, or a control peptide, MuIFNγ (95–125) (5 μM), were added to the cells. After 48 h of incubation, the cell extracts were assayed for relative luciferase activities using a luminometer. D, pJAK2(1001–1013) enhances immune response to BSA in C57BL/6 mice. Mice were injected i.p. with 200 μg of lipo pJAK2(1001–1013), control IFN-γR (IFNGR1) peptide lipo IFNGR1 (253–287) or PBS on day −2, −1, and 0 relative to i.p. injection of 50 μg of BSA. Four weeks later, spleens were removed, and separated cells (5 × 10^5) were stimulated in 96-well plates with 0.5 μg of BSA for 96 h. Tritiated thymidine was added 18 h before harvesting. Triplicates were counted for incorporation of radioactivity and data were represented as cpm.
respectively. pJAK2(1001–1013) alone did not activate the reporter gene (data not shown) and control peptide did not enhance IFN-γ activation of the reporter gene. Thus, consistent with the anti-SOCS-1 effects of pJAK2(1001–1013), the peptide also enhanced IFN-γ function at the level of gene activation.

It has recently been shown that suppression of SOCS-1 in dendritic cells by small-interfering RNA (siRNA) enhances antitumor immunity (27). Therefore, we treated C56BL/6 mice with lipo-dendritic cells by small-interfering RNA (siRNA) enhances antitumor immunity. We have shown here, therefore, that pJAK2(1001–1013) antagonizes SOCS-1 activity at four different levels: IFN-γ antiviral function, IL-6 signal transduction, IFN-γ activation of reporter gene via the GAS promoter, and enhancement of Ag-specific proliferation.

**SOCS-1 protein competes with SOCS1-KIR for binding to pJAK2(1001–1013)**

To directly compare SOCS-1 and SOCS1-KIR for their relative ability to compete for the autophosphorylation peptide pJAK2(1001–1013), S9 insect cells were transfected with the SOCS-1 gene via a baculovirus expression vector for production of SOCS-1 protein (Invitrogen Life Technologies). As shown in Fig. 8, soluble SOCS-1 protein specifically competed with SOCS1-KIR for binding to biotinylated pJAK2(1001–1013), suggesting that SOCS1-KIR recognizes pJAK2(1001–1013) similarly to SOCS-1. This does not preclude other sites on SOCS-1 from interacting with the JAK2 autophosphorylation site. Nor does it preclude SOCS1-KIR from interacting with the catalytic site of JAK2 as has been proposed (15). Additionally, the demonstration of SOCS-1 competition for pJAK2(1001–1013) is consistent with pJAK2(1001–1013) antagonism via sequestration of critical functional site(s) on SOCS-1.

**Discussion**

SOCS-1 is absolutely essential for survival of the individual. Although SOCS-1 knockout mice appear to be normal at birth, they exhibit stunted growth and die neonatally by 3 wk of age (28). These mice exhibit a syndrome characterized by severe lymphopenia, activation of T lymphocytes, fatty degeneration and necrosis of the liver, hemopoietic infiltration of multiple organs, and high levels of constitutive IFN-γ as well as abnormal sensitivity to IFN-γ (Refs. 12 and 28; reviewed in Ref. 29). IFN-γ plays a central role in the pathology as SOCS-1 knockout mice that are deficient in IFN-γ or IFN-γR do not die as neonates. Similar pathology and lethality is observed in normal neonates that are injected with IFN-γ.

The dynamics of induction of SOCS-1 by IFN-γ in cells and the activation of STAT1α is illustrative of how SOCS-1 antagonizes IFN-γ functions under physiological conditions. For example, treatment of monocytes or astrocytes with IFN-γ was followed by activation of the SOCS-1 gene at ~90 min (30, 31). Low doses of IFN-γ resulted in transient increases in SOCS-1 mRNA that returned to baseline after 4 h, whereas high concentrations of IFN-γ resulted in increases of SOCS-1 mRNA up to 24 h. Thus, the SOCS-1 response appears to be induced by the IFN-γ signal. Treatment of hepatocytes from SOCS-1−/− mice with IFN-γ resulted in STAT1α activation within 15 min, which peaked by 2 h before declining (31). Although STAT1α is similarly activated in SOCS-1-deficient livers, it persists for 8 h. SOCS-1 thus appears to attenuate IFN-γ-persistent activation of STAT1α, which nonetheless allows the beneficial effects of IFN-γ-induced signaling.

Given the critical importance of SOCS-1 in modulating the activities of IFN-γ and other inflammatory cytokines that use tyrosine kinases such as JAK2 in their signaling pathways, we have developed the small Tkip, which is a mimetic of SOCS-1 (14). Tkip was designed to recognize the autophosphorylation site on JAK2 involving residues 1001–1013 containing the critical tyrosine at 1007 (15). We showed that Tkip blocked JAK2 autophosphorylation as well as tyrosine phosphorylation of substrates affecting the tyrosine kinase function of c-Src and vascular endothelial growth factor receptor. We subsequently showed that Tkip blocked IL-6-induced activation of the STAT3 oncogene in LNCaP prostate cancer cells, which involved inhibition of JAK2 activation (16). These studies presented a proof-of-concept demonstration of a peptide mimetic of SOCS-1 that regulates JAK2 tyrosine kinase function.

Because of its potential for regulation of inflammatory conditions where tyrosine kinases such as JAK2 play a role in the resultant pathology, we tested Tkip in a mouse model of multiple sclerosis called EAE (17). SJL/J mice were immunized with MBP to induce the relapsing/remitting form of EAE. Tkip, 63 μg every other day, completely protected the mice against relapses every other day, completely protected the mice against relapses compared with control groups in which >70% of the mice relapsed after primary incidence of disease. Protection of mice correlated with lower MBP Ab titers in Tktp-treated groups as well as suppression of MBP-induced proliferation of splenocytes taken from EAE-afflicted mice. Consistent with its JAK2 inhibition function, Tkip also inhibited the activity of inflammatory cytokine TNF-α, which uses the STAT1α transcription factor. Thus, Tkip,
as with SOCS-1, possesses anti-inflammatory activity that protects mice against ongoing relapsing/remitting EAE.

Our design of Tkip was independent of any knowledge of the structural and functional domains of SOCS-1. However, given that we focused on Tkip binding to the autophosphorylation site of JAK2, we compared it with regions of SOCS-1 that have been proposed to be either directly involved in such binding or to be involved in enhancement of SOCS-1 binding to the autophosphorylation site. Yasukawa et al. (15) identified three regions that were directly involved in SOCS-1 binding to JAK2, the large SH2 domain, a N-terminal 12-aa sequence called extended SH2 (ESS), and an additional N-terminal 12-aa region called the KIR. The ESS and SH2 domains were felt to bind to the autophosphorylation or activation site of JAK2, while KIR bound to the catalytic site in this model. The 12-aa ESS sequence consists of residues 68–79, whereas our SOCS1-KIR sequence compared with the KIR above consists of residues 53–68, sharing just the I68 with the ESS and containing three additional residues in its N terminus. In comparative binding, our SOCS1-KIR bound to pJAK2(1001–1013), whereas ESS failed to bind (Fig. 6). Close examination of N-terminal truncated SOCS-1–expressed proteins, designated dN51 (missing residues N-terminal to 51) and dN68, showed that removal of the KIR resulted in loss of binding to JAK2 in transfected cells (15). Furthermore, in direct binding to the pJAK2 autophosphorylation site peptide by truncated dN56 SOCS-1 protein, our SOCS1-KIR sequence except for residues 53–55, was present along with ESS and SH2 (15). Although the region of SOCS-1 that binds to the autophosphorylation site of JAK2 as per Yasukawa et al. (15) is referred to as SOCS-1 SH2 plus ESS, the bindings that they refer to also contained our SOCS1-KIR sequence. Additionally, SOCS-1 protein competed with SOCS1-KIR for pJAK2(1001–1013), suggesting that the two recognized the JAK2 autophosphorylation site similarly. Thus, we feel that the binding data with our SOCS1-KIR are consistent with the binding studies of these authors. It should be noted that the peptide-binding approach used here does not involve assessment of collaboration and/or synergism among the KIR, ESS, and the SH2 domains of SOCS-1. Thus, based on the studies reported here, along with those by others (15), KIR, ESS, and SH2 may all be involved in binding to the JAK2 autophosphorylation site. It remains to be determined as to the extent of their relative roles.

We have shown in this study that SOCS1-KIR, independent of other domains of SOCS-1, can bind directly to a peptide, JAK2(1001–1013), that corresponds to the autophosphorylation site of JAK2. Furthermore, we showed that SOCS1-KIR competed with Tkip for binding to JAK2(1001–1013). The competitions suggest that the peptides recognized JAK2 similarly but not exactly the same way. Phosphorylation of Tyr1007 on the JAK2 peptide enhanced binding of Tkip and SOCS1-KIR. Tkip blocked JAK2 autophosphorylation as well as JAK2 phosphorylation of STAT1α, while SOCS1-KIR did not block autophosphorylation but did block phosphorylation of STAT1α, similar to the pattern or profile of SOCS-1 inhibition of phosphorylation (12). The peptides were also functionally similar in inhibiting IFN-γ activation of macrophages to produce NO and inhibition of Ag-specific induction of proliferation of splenocytes, with Tkip being the more effective inhibitor. Thus, we have shown here that the KIR region of SOCS-1 can directly bind to the autophosphorylation site of JAK2. We have also further demonstrated the SOCS-1 mimetic property of Tkip at the level of JAK2 recognition.

Tkip is relatively hydrophobic, while SOCS1-KIR is hydrophilic. However, both peptides have hydrophobic profiles that are complementary to that of pJAK2(1001–1013). ESS, however, had a hydrophobic profile different from Tkip. Tkip was designed to have a hydrophobic profile complementary to that of pJAK2(1001–1013) (14, 32). Thus, Tkip would recognize primarily hydrophobic residues or groups in the pJAK2 peptide, while SOCS1-KIR would recognize primarily hydrophilic residues or groups. The binding competition could thus be due to a steric interference, which is consistent with differential effects of Tkip and SOCS1-KIR on JAK2 kinase activity.

The fact that the KIR of SOCS-1 can bind directly to pJAK2(1001–1013) raises the possibility that pJAK2(1001–1013) can function as an antagonist of SOCS-1. We have thus shown here under four different types of experiments that pJAK2(1001–1013) possesses SOCS-1 antagonist properties. First, pJAK2(1001–1013) enhanced suboptimal IFN-γ activity. Second, prostate cancer cells transfected for constitutive production of SOCS-1 protein had reduced activation of STAT3 by IL-6 treatment. pJAK2(1001–1013) reversed the SOCS-1 effect. Third, pJAK2(1001–1013) enhanced IFN-γ activation of the luciferase reporter gene via the GAS promoter. Fourth, pJAK2(1001–1013) enhanced Ag-specific splenocyte proliferation. As indicated above, treatment of cells with IFN-γ resulted in activation of the SOCS-1 gene in ~90 min and it has been proposed that it is associated with the physiological attenuation of the IFN-γ response by SOCS-1 (30, 31). Consistent with this, it has been reported recently that siRNA inhibition of SOCS-1 expression in bone marrow dendritic cells resulted in enhanced CTL activity and IFN-γ production by ELISPOT, culminating in enhancement of antitumor immunity (27).

We have thus shown here that SOCS1-KIR binds directly to the autophosphorylation site of JAK2, similar to the binding of Tkip SOCS-1 mimetic, which results in inhibition of JAK2 phosphorylation of substrate. This directly identifies a region of SOCS-1 that possesses intrinsic antikinase function. Related to this, the autophosphorylation site peptide, pJAK2(1001–1013), functioned as an antagonist of SOCS-1. These findings with SOCS-1 mimetics and antagonists have implications for novel therapeutic approaches to mimicking SOCS-1 for treatment of inflammatory diseases and for suppressing SOCS-1 to enhance the immune response against cancer and infectious diseases.

Disclosures

The authors have no financial conflict of interest.

References

SOCS-1 MIMETIC AND ANTAGONIST


