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Characterization of a Peptide Inhibitor of Janus Kinase 2 That Mimics Suppressor of Cytokine Signaling 1 Function

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Positive and negative regulation of cytokines such as IFN-γ are key to normal homeostatic function. Negative regulation of IFN-γ in cells occurs via proteins called suppressors of cytokine signaling (SOCS)1 and -3. SOCS-1 inhibits IFN-γ function by binding to the autophosphorylation site of the tyrosine kinase Janus kinase (JAK2). We have developed a short 12-mer peptide, WLVFFVIFYFFR, that binds to the autophosphorylation site of JAK2, resulting in inhibition of its autophosphorylation as well as its phosphorylation of IFN-γ receptor subunit IFNγR-1. The JAK2 tyrosine kinase inhibitor peptide (Tkip) did not bind to or inhibit tyrosine autophosphorylation of vascular endothelial growth factor receptor or phosphorylation of a substrate peptide by the protooncogene tyrosine kinase c-src. Tkip also inhibited epidermal growth factor receptor autophosphorylation, consistent with the fact that epidermal growth factor receptor is regulated by SOCS-1 and SOCS-3, similar to JAK2. Although Tkip binds to unphosphorylated JAK2 autophosphorylation site peptide, it binds significantly better to tyrosine-1007 phosphorylated JAK2 autophosphorylation site peptide. SOCS-1 only recognizes the JAK2 site in its phosphorylated state. Thus, Tkip recognizes the JAK2 autophosphorylation site similar to SOCS-1, but not precisely the same way. Consistent with inhibition of JAK2, Tkip inhibited the ability of IFN-γ to induce an antiviral state as well as up-regulate MHC class I molecules on cells at a concentration of ~10 μM. This is similar to the $K_d$ of SOCS-3 for the erythropoietin receptor. These data represent a proof-of-concept demonstration of a peptide mimetic of SOCS-1 that regulates JAK2 tyrosine kinase function. The Journal of Immunology, 2004, 172: 7510–7518.

Tyrosine kinases play an important role in both normal and abnormal cell function (reviewed in Ref. 1). Uncontrolled or constitutive tyrosine kinase activity can result in diseases such as cancer and inflammatory disorders associated with inflammatory or Th1 lymphocytes (1, 2). Many oncogenes code for proteins that are tyrosine kinases (1). The targeted approach of treatment of cancer is directed toward development of specific tyrosine kinase inhibitors (reviewed in Ref. 3). The most successful example of targeted therapy against cancer is the identification of the drug Gleevec against chronic myelogenous leukemia (reviewed in Ref. 4). This cancer is due to chromosomal rearrangements where the p210 BCR-Ab1 tyros phosphorylation site of tyrosine kinase is rendered constitutively active. The chemical Gleevec binds to the ATP binding site of this kinase and inhibits its kinase activity (4). This results in almost total control of chronic myelogenous leukemia without the undesirable side effects associated with conventional chemotherapy (4).

The Janus kinase (JAK)$^3$ family of tyrosine kinases were first described for their role in signaling through the IFN receptors of both type I and type II IFNs (reviewed in Ref. 5). Among the IFNs, JAK2 is associated with the type II IFN, IFN-γ (5). The immediately early signal transduction events associated with IFN-γ’s interaction with its receptor involves the obligatory action of two tyrosine kinases, JAK1 and JAK2 (5). The IFN-γ receptor (IFNγR) system is a heterodimeric complex consisting of an α subunit, IFNγR-1, and a β subunit, IFNγR-2, both of which are essential for biological activities of IFN-γ (5). JAK1 is constitutively associated with the IFNγR-1 chain, whereas JAK2 is associated with the IFNγR-2 chain (5).

Interaction of IFN-γ, primarily with the IFNγR-1 subunit, initiates a sequence of events that results in increased binding of JAK2 to IFNγR-1 (5). This interaction has important consequences for subsequent critical phosphorylation events (5). JAK2, in the process of binding to IFNγR-1, undergoes autophosphorylation, and at the same time IFNγR-1 is phosphorylated. These events, occurring in concert with JAK1 function, result ultimately in recruitment and tyrosine phosphorylation of the IFN-γ transcription factor STAT1α (5). The activity of JAK tyrosine kinases, and consequently signaling via the JAK/STAT pathway, is controlled negatively by members of the suppressors of cytokine signaling (SOCS) family, also called the cytokine-inducible SH2-containing (CIS) family (6–9). These inducible proteins are of significantly varied lengths, but share domains of homology that characterize the family and their function.

There are a variety of approaches to the regulation of specific tyrosine kinases that are associated with cancer and inflammatory disorders like arthritis. These involve receptor-specific Abs, decoy receptors to bind and inactivate ligands, small molecules with specificity toward the ATP-binding sites, and small molecules that block kinase function by unknown mechanisms (3, 10). The classic endothelial growth factor, CDK, cyclin-dependent kinase; Lapo-Tkip, lipophilic Tkip; R-PE, R-phycoerythrin; EGF, epidermal growth factor; KIR, kinase inhibitory region.

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and original demonstration of a mAb that blocked a tyrosine kinase function is Herceptin, which is a mAb specific for the Her-2 receptor (10). This discovery has resulted in the treatment of aggressive forms of breast cancer where the cancer cells express the oncoprote Her-2.

As indicated above, Gleevec is the best example of a small drug identified for the treatment of cancer, where it binds to the ATP-binding site of the oncoprote BCR-Abl (4). We have synthesized a small peptide inhibitor of JAK2. Unlike Gleevec, this peptide was developed to recognize the autophosphorylation site on JAK2, and thus represents a novel approach to inhibition of tyrosine kinase activity. In this study, we describe the specificity of the JAK2 kinase inhibitor in terms of tyrosine phosphorylation and recognition of the autophosphorylation site of JAK2 and related kinases. The effect of the peptide inhibitor on IFN function was also determined, because JAK2 is important in the signal transduction mechanism of IFN-γ. Finally, we interpret the function of the JAK2 inhibitor in the context of SOCS with particular emphasis on SOCS-1, which inhibits JAK2 function via binding to its autophosphorylation site.

Materials and Methods

Cell culture and virus

All cell lines were from American Type Culture Collection (Manassas, VA). WEHI-3 murine macrophages were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (HyClone, Logan, CT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 M 2-ME. WISH human fibroblast cells were maintained in Eagle’s MEM (JRH Biosciences, Lenexa, KS) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) in six-well tissue culture plates at 37°C in a 5% CO₂ atmosphere. Bovine aortic endothelial cells (BAECs) were generously provided by Drs. R. Johnson and T. Nakagawa of the Division of Nephrology, College of Medicine, University of Florida. BAEC culture reagents were purchased from Cambrex (Walkersville, MD). BAECs were cultured according to the manufacturer’s instructions in six-well tissue culture plates at 37°C in a 5% CO₂ atmosphere. Starvation medium contains Eagle’s MEM without antibiotics. Encephalomyocarditis virus (EMCV) was obtained from American Type Culture Collection and stored at ~70°C until use.

Peptides

The peptides used in the current study are listed in Table I, and are synthesized in our laboratory on an Applied Biosystems (Foster City, CA) 9050 automated peptide synthesizer using conventional fluorenlyl oxybenzyl chemistry as described previously (11). The addition of a lipophilic group (palmitoyl-lysine) to the N terminus of a synthetic peptide was performed as the last step using a semiautomated protocol. Peptides were characterized by mass spectrometry and purified by HPLC. Peptides were dissolved in either deionized water or DMSO (Sigma-Aldrich, St. Louis, MO).

Binding assays

For ELISA binding assays, tyrosine kinase inhibitor peptide (Tkip) and other peptides to be tested were bound to 96-well plates in 0.1 M carbonate binding buffer (pH 9.6) at a final concentration of 3 μg/well (50 μl). Wells were then washed three times with wash buffer containing 0.9% NaCl and 0.05% Tween-20 in PBS and blocked with 2% gelatin and 0.05% Tween-20 in PBS for 1 h at room temperature. Wells were then washed three times with wash buffer and incubated with various concentrations of biotinylated JAK2 wild-type (WT) peptide and biotinylated JAK2 (p-JAK2) WT peptide for 1 h at room temperature in blocking buffer. Following incubation, wells were washed five times with wash buffer to remove any unbound biotinylated peptides. Bound biotinylated peptides were detected by incubation with a 1/500 dilution of Neutrasvidin-biotin binding protein conjugated with HRP (Molecular Probes, Eugene, OR) in blocking buffer for 1 h at room temperature. Wells were then washed five times with wash buffer and developed with a solution of o-phenylenediamine in stable peroxidase buffer (Pierce, Rockford, IL). The assay was stopped with the addition of 2 M H₂SO₄ (50 μl) to each well. Absorbance was measured using a 450 microplate reader (Bio-Rad, Hercules, CA) at 490 nm. Control experiments were conducted as described above in the absence of immobilized peptides.

Peptide competition experiments were conducted using peptides derived from JAK2, vascular endothelial growth factor (VEGF)R, and cyclin-dependent kinase (CDK) to compete with biotinylated JAK2 WT peptide for binding to Tkip. Binding of biotinylated JAK2 WT was determined as above, except that following Tkip immobilization, washing, and blocking in blocking buffer for 1 h at room temperature, unlabeled competitors were added to wells and incubated for 30 min at varying concentrations before biotinylated JAK2 WT peptide was added. Detection of binding was conducted as described above.

Immunoblot analysis

WISH fibroblast cells were plated in six-well plates at a cell density of 3 × 10⁶ cells/well. After overnight incubation with complete culture medium, WISH cells were incubated in starvation medium for 17 h and pretreated with complete culture medium or different concentrations of Tkip (8 or 1 μM) for an additional 17 h at 37°C in a 5% CO₂ atmosphere. WISH cells were then incubated in the presence or absence of 5000 U/ml IFN-γ (PBL Biomedical Laboratories, Piscataway, NJ) to activate the JAK-STAT pathway. Fibroblast cells were washed twice in cold PBS to remove medium and cell debris. Cell lysates were prepared by adding 200 μl of cold lysis buffer (50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 2 mM EGTA, 2 mM EDTA, 50 mM NaF, 2 mM Na₃VO₄, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM PMSF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS) to each well. Lysis was allowed to proceed for 1 h at 4°C (rocking) to ensure complete lysis. Lysates were then centrifuged to remove cell debris, and the supernatant was transferred to a fresh microcentrifuge tube. Samples containing lysis, lysis buffer, and sample buffer were boiled for 5 min and pulse centrifuged. Protein lysates were resolved by SDS-PAGE on a 12% polyacrylamide gel (Bio-Rad). Proteins were then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ) overnight at low voltage. To reduce nonspecific binding, membranes were incubated in blocking buffer containing 5% nonfat dry milk in PBS for 1 h at room temperature and washed in wash buffer containing 1% nonfat dry milk and 0.1% Tween-20 in PBS (PBST) three times. To detect phosphorylated STAT1, membranes were incubated with Abs to tyrosine-phosphorylated STAT1 (Cell Signaling Technologies, Beverly, MA), phosphorylated at tyrosine residue 701 (1:1000) in wash buffer overnight with agitation at 4°C. After three washes in PBST, membranes were incubated in HRP-conjugated goat anti-rabbit IgG secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1/5000 in wash buffer for 1 h at room temperature. Following three washes in wash buffer, membranes were incubated for 1 min with ECL detection reagents (Amersham Biosciences) and exposed to photographic film to visualize protein bands.

Flow cytometry

WISH fibroblast cells (2 × 10⁶) were incubated for 48 h with medium alone, 25 μM MuIFN-γ/905,125 peptide, or lipophilic Tkip (Lipo-Tkip) at varying concentrations (1, 10, 25 μM) in the presence or absence of 5000 U/ml IFN-γ in six-well culture plates at 37°C in a 5% CO₂ atmosphere. Following incubation, cells were washed twice with PBS and harvested by trypsinization into two sets of 5-ml round-bottom polystyrene tubes (Fisher, Pittsburgh, PA) and washed twice with PBS. For cell surface staining, a direct immunofluorescence protocol was employed. Briefly, cells were incubated on ice with a 100-μl staining solution in PBS of either anti-human MHC class I mAb conjugated to R-phycocerythrin (R-PE; 1:100) or with monoclonal mouse IgG2a Ab conjugated to R-PE (1:100) as an isotype control for 1 h at room temperature in the absence of light.

Table I. Amino acid sequences of the peptides used in this study

| Peptide | Sequence |
|---------|----------|
| Tkip    | WLVFFVYFFR |
| JAK2 WT | 100 LPCQDEKXVYKREP |
| p-JAK2 WT | 100 LPCQDKHVYKREP |
| VEGF    | 1205 GSDGVYQANFKM |
| CDK-2 cyclin box | 4KTEGVPSAIREISLLKELNI |
| MuIFN-γ/905,125 | 100 AKFEVNQFQRGAFNLRVQHLLPESSL |

* Peptides were synthesized as described in Materials and Methods. Murine IFN-γ sequence is derived from the mature form. Lipophilic group and biotinylated modifications were added to the N terminus of the peptide. Tyrosines targeted for phosphorylation are indicated in bold. The square denotes the phosphorytoiyne motif. The JAK2 WT sequence is the same for both mice and humans.
fluorescent-conjugated isotype Ab was used to determine background fluorescence by identifying nonspecific binding of the mAb to WISH cells. The above Abs were purchased commercially from Ancell (Bayport, MN). Following incubation, cells were then washed three times with PBS to remove unbound Ab molecules. WISH fibroblast cells were finally resuspended in the R-PE-labeled cells. For each sample, 10,000 stained cells were examined. Flow cytometry data were analyzed using CellQuest analysis software (BD Biosciences).

**Immunoprecipitation**

BAECs were plated at a density of 3 x 10^5/well in six-well plates and allowed to incubate for 10 h at 37°C. Growth medium was then removed and replaced with growth medium with or without peptides at the indicated concentrations overnight at 37°C. Cells were then treated with serum-free medium alone or serum-free medium containing peptides at the indicated concentrations for 2 h. BAECs were then incubated in the presence or absence of 50 ng/ml VEGF (Upstate Biotechnology, Lake Placid, NY) in serum-free medium for 10 min and lysed with 500 μl lysis buffer. Lysates were then boiled (100°C) for 10 min, and 20 μl of each lysate was added to 2 μl of JAK2 agarose beads (Upstate Biotechnology), 1 μl of μg [γ-32P]ATP (Amersham Biosciences), and either Tkip or JAK2 WT peptide incubated in kinase buffer (10 mM HEPES (pH 7.4), 30 mM sodium chloride, 0.1 mM sodium orthovanadate, 5 mM magnesium chloride, and 5 mM manganese chloride). JAK2 kinase assays were performed in 35-μl reaction volumes containing 10.5 μl of Tkip, 20 μl of JAK2 agarose beads, 1 μl of soluble IFNγR-1, and 3.5 μl of [γ-32P]ATP incubated at 30°C for 30 min with intermitent agitation. It was determined that addition of a mouse soluble IFNγR-1 subunit dramatically stimulated JAK2 kinase activity and, hence, was added at 2 μg per reaction. EGFR, VEGFR, and JAK2 kinase reactions were terminated with the addition of 5 μl of 6× SDS sample buffer (0.5 M Tris-HCl (pH 6.8), 36% glycerol, 10% SDS, 9.3% DTT, and 0.012% bromophenol blue). Incubation of JAK2 agarose beads in SDS sample buffer (100°C) was designed to elute bound proteins from the agarose beads. The reaction mixtures were separated on a 10% SDS polyacrylamide gel. Autoradiography was used to determine phosphorylation activity.

**Antiviral assay**

Antiviral activity was determined using a standard viral cytopathogenic effect assay described previously with minor modifications (12). Antiviral assays were performed to evaluate the ability of Tkip to block antiviral activity mediated by IFN-γ. Briefly, WEHI-3 murine macrophage cells (5 x 10^5) were plated into 6-well plates (BD Labware, Franklin Lakes, NJ) at 37°C in a 5% CO2 atmosphere. Following incubation, WEHI-3 cells were washed three times with PBS to remove non-adherent cells. Supernatants were then washed three times with PBS to remove virus particles and incubated in fresh growth medium for an additional 24 h at 37°C. Plates were subsequently blotted dry and stained with 0.1% crystal violet solution for 5 min to stain live cells. Unbound crystal violet was aspirated, and the plates were thoroughly rinsed with deionized water, blotted, and allowed to air dry. Plates were then scanned using an Astra 2100U flatbed computer scanner (UMAX Technologies, Dallas, TX) and analyzed using ImageJ 1.29 software (National Institutes of Health) to assess cell survival. Percentages of cell survival were determined by comparing experimental treatment groups with the virus-only control group. Recombinant murine IFN-γ (specific activity, 1 x 10^7 U/ml) used in the antiviral assay described above was obtained from PBL.

**In vitro kinase assays**

Autophosphorylation activity of epidermal growth factor (EGFR) and VEGFR-1 was measured in a reaction mixture containing kinase buffer (20 mM Tris-HCl (pH 7.5), 2 mM DTT, 50 mM potassium chloride, 0.3 mM sodium orthovanadate, 5 mM magnesium chloride, 10 mM glycerophosphate, 2 mM EGTA, and 1 M manganese chloride), Tkip, substrates, and 5 μCi of [γ-32P]ATP (specific activity, 6000 Ci/mmol; 1 μCi = 37 MBq) (Amersham Biosciences). EGFR and VEGFR in vitro kinase assays were conducted in 22.5-μl reaction volumes containing 10 μl of Tkip, 2.5 μl of substrates, and 10 μl of [γ-32P]ATP incubated at 30°C for 10 min. EGFR (supplied precomplexed with EGF) was obtained from Upstate Biotechnology, VEGFR- and VEGF were obtained from Calbiochem (San Diego, CA) and PeproTech (Rocky Hill, NJ), respectively.

The autophosphorylation assay was performed in reaction mixtures containing recombinant human JAK2 immobilized on agarose beads (Upstate Biotechnology), 1 μg/ml of [γ-32P]ATP (Amersham Biosciences), and either Tkip or JAK2 WT peptide incubated in kinase buffer (10 mM Hepes (pH 7.4), 30 mM sodium chloride, 0.1 mM sodium orthovanadate, 5 mM magnesium chloride, and 5 mM manganese chloride), JAK2 kinase assays were performed in 35-μl reaction volumes containing 10.5 μl of Tkip, 20 μl of JAK2 agarose beads, 1 μl of soluble IFNγR-1, and 3.5 μl of [γ-32P]ATP incubated at 30°C for 30 min with intermitent agitation. It was determined that addition of a mouse soluble IFNγR-1 subunit dramatically stimulated JAK2 kinase activity and, hence, was added at 2 μg per reaction. EGFR, VEGFR, and JAK2 kinase reactions were terminated with the addition of 5 μl of 6× SDS sample buffer (0.5 M Tris-HCl (pH 6.8), 36% glycerol, 10% SDS, 9.3% DTT, and 0.012% bromophenol blue). Incubation of JAK2 agarose beads in SDS sample buffer (100°C) was designed to elute bound proteins from the agarose beads. The reaction mixtures were separated on a 10% SDS polyacrylamide gel. Autoradiography was used to determine phosphorylation activity.

Ssrc kinase activity was performed with a Src substrate peptide (KVEKGEGTVGVVK) used in a Src kinase assay kit (Upstate Biotechnology) according to the manufacturer’s specifications. Briefly, Src substrate peptide was incubated in Src kinase buffer (100 mM Tris-HCl (pH 7.2), 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 0.02 mM Na2VO4, and 2 mM DTT), 5 μCi of [γ-32P]ATP (75 mM MnCl2 and 500 μM ATP), recombinant human Src (Upstate Biotechnology), and Tkip. JAK2 WT peptide, or in the absence of peptide for 10 min at 30°C. Reaction mixtures were spotted on P81 phosphocellulose discs (supplied with kit) to bind phosphorylated Src substrate peptide, washed three times for 5 min with 0.75% phosphoric acid, washed twice with acetone for 1 min, and placed in vials to which 4 ml of ScintiVerse (Fisher, Pittsburgh, PA) was added. The phosphocellulose discs were analyzed using a liquid scintillation counter to measure [32P]-labeled proteins. For comparison with Src, JAK2 was also assayed using the same procedure. The assay was set up as described above for the Src kinase assay. However, reactions were incubated in the presence of 2 mM DTT in the buffer to release JAK2 from the agarose beads. Following the appropriate incubation period, reactions were gently centrifuged. Supernatants were spotted on phosphocellulose discs and processed as above. Unless otherwise stated, the peptides used in the kinase reactions described above were used at 50 μM. Immunoblotting was performed in parallel to determine protein levels used in the kinase reactions.

**Results**

The autophosphorylation site of human JAK2 consists of residues 1010LPQDEKEYVKVEP with 1010Y as the tyrosine autophosphorylation residue that results in activation of JAK2 (13). We focused on the complementary peptide approach to develop a short peptide capable of binding to this site (14). We have used this approach to develop peptides that bind to the neupeptide arginine vasopressin (15). The complementarity refers to the hydrophobic complementarity, which has been shown empirically to result in peptide/peptide binding (14). Recently, an algorithm has been developed that specifies the best complementarity fit (16). We generated the sequences of several peptides that varied in their complementarity to 1010LPQDEKEYVKVEP. We discovered that the best complementarity fit did not necessarily result in the best binding to JAK2 peptide (data not shown). Thus, the best binding, which was not the best complementarity fit, occurred with complementarity peptide WLVFFVIFIVYFR. This peptide was developed by reading the complementary strand codons in the JAK2 autophosphorylation site in the 5′-3′ direction as originally described in detail by others (17). Data on binding of WLVFFVIFIVYFR to 1010LPQDEKEYVKVEP, as determined by ELISA, are presented in the article. The sequences of the peptides used are presented in Table I. Biotinylated JAK2 autophosphorylation peptide (biotinylated JAK2
WT) was added at different concentrations to solid-phase complementarypeptide, designated Tkip, as well as to solid-phase control peptides. These control peptides consisted of IFN-γ sequence 95–125 (IFN-γ95–125), CDK2 cyclin box peptide CDK41–61, and VEGFR autophosphorylation peptide VEGFR1208–1222. As shown in Fig. 1A, JAK2 WT bound only to Tkip in a dose-dependent manner. Binding to the control peptides was negligible. In ELISA competitions, JAK2 WT, but neither CDK41–61 nor VEGFR1208–1222, inhibited biotinylated JAK2 WT binding to Tkip (Fig. 1B). The binding data suggest that the Tkip peptide specifically recognized JAK2 WT.

We next determined whether Tkip could inhibit JAK2 autophosphorylation as well as phosphorylation of IFN-γ receptor subunit IFNGR-1. As shown in Fig. 2A, Tkip at 50 μM inhibited both the autophosphorylation of JAK2 as well as JAK2 phosphorylation of IFNGR-1. A control peptide, JAK2 WT, at the same concentration had no effect on JAK2-induced tyrosine phosphorylations. Thus, consistent with Tkip binding to JAK2 WT, it also inhibited JAK2 autophosphorylation as well as JAK2 phosphorylation of IFNGR-1.

FIGURE 1. Binding of Tkip by JAK2 autophosphorylation peptide, JAK2 WT. A. Direct binding of WT JAK2 peptide (see Table I for sequences) to Tkip. The WT JAK2 peptide was synthesized with a biotin group incorporated at its N terminus during peptide synthesis, and the peptide was purified. Biotinylated JAK2 WT peptide, at the indicated concentrations, was added in triplicate to wells of 96-well plates coated with either Tkip, VEGFR peptide, CDK-2 cyclin box peptide, or MuIFN-γ95–125 peptide (see Table I for sequences). Wells were blocked with PBS plus 2% gelatin plus 0.1% Tween 20. The assay was developed using standard ELISA methods, using a neutravidin-HRP conjugate to detect bound biotinylated WT JAK2. Non-specific binding was determined from wells that were not coated with any peptide to which the same concentrations of biotinylated peptide were added. B. Biotinylated WT JAK2 peptide was bound to Tkip coated on 96-well plates, either in the absence (100% binding) or presence of indicated concentrations of JAK2 WT peptide, VEGFR peptide, or an unrelated peptide (MuIFN-γ95–125). Bound biotinylated WT JAK2 peptide was detected by ELISA using a neutravidin-HRP conjugate. The data are representative of at least two separate experiments.

Tkip was next tested for its inhibitory effects against several other tyrosine kinases. VEGFR is involved in the development and growth of the vascular endothelial system (18). As shown in Fig. 2B, Tkip at 50 μM did not inhibit the autophosphorylation of VEGFR, but under the same conditions completely inhibited JAK2 autophosphorylation as well as JAK2 phosphorylation of IFNGR-1 (1F). Thus, compared with VEGFR, Tkip shows specificity toward JAK2. This is consistent with the failure of Tkip to bind to VEGFR autophosphorylation site as per Fig. 1.

We also tested Tkip against a nonautophosphorylation tyrosine kinase, c-src. As shown in Fig. 2C, Tkip at 50 μM failed to inhibit c-src phosphorylation of a protein substrate. By contrast, Tkip significantly blocked JAK2 autophosphorylation/IFNGR-1 phosphorylation as estimated by >95% inhibition of 32P incorporation into JAK2/IFNGR-1 (Fig. 2D). Thus, the data on Tkip failure to block VEGFR and c-src tyrosine phosphorylations are evidence of specificity of Tkip for inhibition of JAK2 autophosphorylation via interaction with the JAK2 autophosphorylation site.

Interaction of Tkip with the JAK2 autophosphorylation site and inhibition of JAK2 function raises the question of possible functional relationship of Tkip to a group of regulators called SOCS. SOCS are recently discovered negative regulators of cytokine, growth factors, and hormone signaling (reviewed in Refs. 6–9).

Currently, there are eight identified members of the SOCS family, SOCS-1 to -7 and CIS. SOCS-1 and -3 are of interest to the current studies, because they are the negative regulators of both JAK2 and the EGFR (6–9, 19). We therefore determined whether Tkip could inhibit EGFR tyrosine kinase activity. As shown in Fig. 2E, Tkip at 50 μM completely inhibited EGFR autophosphorylation. For comparison, Tkip also inhibited JAK2 autophosphorylation as well as JAK2 phosphorylation of IFNGR-1 (Fig. 2F). Thus, Tkip inhibited EGFR autophosphorylation, which is consistent with its specificity for the SOCS-1 and -3 autophosphorylation sites of JAK2 and EGFR. It should be kept in mind that EGFR autophosphorylation is complex, with up to five autophosphorylation sites (20). Thus, just how SOCS-1 and -3 recognize EGFR is currently not known.

We next compared Tkip inhibition of JAK2 and EGFR autophosphorylation in a dose-response study. As shown in Fig. 3, Tkip similarly inhibited autophosphorylation of JAK2 (Fig. 2A) and EGFR (Fig. 2B), with 25–50 μM significantly blocking JAK2 autophosphorylation, and 12–25 μM blocked IFNGR-1 phosphorylation by JAK2, whereas 6–12 μM significantly blocked EGFR phosphorylation. Thus, the patterns of dose-response inhibition of JAK2 and EGFR were similar. It is of interest that Tkip inhibited JAK2 phosphorylation of IFNGR-1 at a lower concentration than that for JAK2 autophosphorylation itself. This suggests that Tkip can block JAK2 phosphorylation of a substrate (IFNGR-1) more effectively than the autophosphorylation of JAK2, which may reflect the possibility that Tkip binds p-JAK2 more effectively than it does unphosphorylated JAK2.

The binding data of Fig. 1 involved JAK2 WT that was not phosphorylated at 1007Y. JAK2 WT recognition by SOCS-1 has been shown to involve phospho-1007Y (7). Thus, Tkip does not need phosphorylation of 1007Y to bind to JAK2 WT. However, we were interested in determining the relative binding of Tkip to JAK2 WT unphosphorylated vs phosphorylation at 1007Y (p-JAK2 WT). As shown in Fig. 4, Tkip bound both JAK2 WT and p-JAK2 WT in a dose-dependent manner, but binding was most efficient to p-JAK2 WT. Fifty percent end-point concentrations were ~9-fold lower for p-JAK2 WT binding vs unphosphorylated JAK2 WT binding. Thus, phosphorylation of 1007Y enhances Tkip binding to the JAK2 autophosphorylation site.
FIGURE 2. Kinase inhibitory specificity of Tkip. A, Tkip inhibits autophosphorylation of JAK2. Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring JAK2 autophosphorylation. Kinase reactions were subjected to SDS-PAGE, and the gels were dried. Dried gels were subjected to autoradiography to detect \(^{32}\)P-labeled proteins (upper and middle panels). The negative control peptide was the JAK2 WT peptide used at the same concentration. Genistein, a nonspecific inhibitor of JAK2, was used as a positive control. Total JAK2 protein was measured from separate reactions that were subjected to SDS-PAGE, and the proteins were Western transferred to a nitrocellulose membrane, followed by detection with standard immunoblotting and ECL detection protocols (bottom panel). The data are representative of at least two separate experiments. B, Tkip does not inhibit autophosphorylation of VEGFR. Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring VEGFR autophosphorylation. The control peptide was the JAK2 WT peptide (see Table I) used at the same concentration. Kinase reactions were subjected to SDS-PAGE, and the gels were dried. Dried gels were subjected to autoradiography to detect \(^{32}\)P-labeled proteins (upper panel). Total VEGFR protein was measured from separate reactions that were subjected to SDS-PAGE, and the proteins were Western transferred to a nitrocellulose membrane followed by detection with standard immunoblotting and ECL detection protocols (lower panel). C, Tkip does not inhibit tyrosine phosphorylation activity of c-src. Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring c-src tyrosine phosphorylation of a synthetic substrate peptide. c-src kinase activity was determined using a kit purchased from Upstate Biotechnology. The control peptide (c-src plus Control peptide) was the JAK2 WT peptide used at the same concentration. “None” represents reactions without c-src or peptides as a measure of background. Triplicate samples of the kinase reactions were spotted on P81 cellulose discs, and processed as described by the manufacturer. The discs were counted for radioactivity, and kinase activity is reported as percentage of the activity of the reaction containing neither Tkip nor control peptide (c-src alone), after subtraction of background (None). D, Tkip inhibition of kinase reactions for JAK2 were setup as described in A as a positive control, but samples were processed as in C. Activity is reported as percentage of activity in reactions containing JAK2 and IFNGR-1 alone, after subtraction of background. E, Tkip peptide inhibits autophosphorylation of EGFR. Tkip peptide was added at 50 μM, where indicated, to in vitro kinase assays measuring EGFR autophosphorylation. The negative control peptide, 50 μM, was the same as in A (JAK2 WT; see Table I). Kinase reactions were subjected to SDS-PAGE, and the gels were dried. Dried gels were subjected to autoradiography to detect \(^{32}\)P-labeled proteins (upper panel). Total EGFR protein was measured from separate reactions that were subjected to SDS-PAGE, and the proteins were Western transferred to a nitrocellulose membrane, followed by detection with standard immunoblotting and ECL detection protocols (lower panel). F, As a positive control, we demonstrate that Tkip inhibited JAK2 in the same experiment. Samples were set up and run as in A. This experiment was run in parallel with that of B and E.
FIGURE 3. Dose response of Tkip inhibition of JAK2, IFNGR-1, and EGFR autophosphorylation in vitro. A, Tkip was incubated with JAK2, IFNGR-1, and [32P]ATP for 30 min at 30°C at the indicated concentrations. The kinase reaction was resolved on a 10% SDS-PAGE. The gel was dried and exposed to photographic film for 1 h at −70°C to detect phosphorylated proteins (upper panel). Kinase reaction mixtures were subjected to immunoblotting with a probe specific for JAK2 and IFNGR-1 as an internal protein loading control (second and fourth panel). B, Tkip was incubated with EGF, EGFR, and [32P]ATP for 10 min at 30°C at the indicated concentrations. The kinase reaction was resolved on a 10% SDS-PAGE. The gel was dried and exposed to photographic film for 1 h at −70°C to detect phosphorylated proteins (upper panel). Kinase reaction mixtures were subjected to immunoblotting with a probe specific for EGFR as an internal protein loading control (lower panel). The data are representative of at least two separate experiments.

It has been firmly established that tyrosine phosphorylation of STAT1α at a specific tyrosine residue (Tyr701) is required for the activation, dimerization, nuclear translocation, and downstream biological effects of IFN-γ stimulation (5). To assess whether Tkip could inhibit intracellular STAT1α activation, we investigated the effect of Tkip on STAT1α tyrosine phosphorylation in human fibroblast WISH cells. Cells were treated with a cell-permeable lipophilic version of Tkip, Lipo-Tkip, and IFN-γ, as indicated in Fig. 5A, and whole-cell lysates were examined using immunoblot analysis with Abs specific for STAT1α and phosphorylated STAT1α. Cells pretreated with 8 μM Tkip for 17 h and subsequently stimulated with 5000 U/ml IFN-γ for 30 min showed complete abolishment of IFN-γ-induced STAT1α tyrosine phosphorylation. A lipophilic irrelevant peptide (MulIFN-γ95–125; 8 μM) was used to show that the results observed were not dependent solely on the lipophilic modification of Tkip. Tyrosine phosphorylation of STAT1α was not affected in cells treated with IFN-γ in the absence of Tkip. STAT1α protein levels in each treatment group were monitored by reprobing the membrane with anti-STAT1α Abs. By contrast, Tkip, under the same conditions, failed to inhibit VEGFR activation in BAECs, as determined by autophosphorylation of VEGFR (Fig. 5B). These results clearly demonstrate the ability of Tkip to inhibit IFN-γ-mediated intracellular phosphorylation of STAT1α at the level of the cell.

Functionally, because Tkip inhibits JAK2 autophosphorylation and subsequent phosphorylation of IFNGR-1, and phosphorylation of STAT1α, one would predict that Tkip would inhibit IFN-γ-induced antiviral activity. Accordingly, we infected WEHI-3 cells with EMCV and protected the cells against EMCV cytopathic effects with 2000 U/ml mouse IFN-γ. Treatment of WEHI-3 cells with 10 μM Lipo-Tkip along with IFN-γ resulted in ~75% reduction in IFN-γ antiviral activity as per increased cytopathogenic effects with 2000 U/ml mouse IFN-γ stimulation (5).

FIGURE 4. Binding of unphosphorylated JAK2 WT peptide vs p-JAK2 WT peptide to Tkip. Various concentrations of biotinylated unphosphorylated JAK2 WT (JAK2 WT) and biotinylated p-JAK2 WT peptides were incubated in the presence of immobilized Tkip. Binding was measured by solid-phase binding assays. The data are representative of two independent experiments performed in triplicate. Binding of p-JAK2 WT peptide vs unphosphorylated JAK2 WT peptide was found to be statistically significant (p < 0.005) by Student’s t test.

FIGURE 5. Tkip inhibits IFN-γ-induced STAT1α activation in WISH cells but does not inhibit VEGF-induced activation of VEGFR in BAECs. A, Serum-starved WISH cells were pretreated with medium alone or Lipo-Tkip (8 or 1 μM) for 17 h. Following 30-min incubation in the presence or absence of 5000 U/ml IFN-γ, cells were washed, harvested, and lysed. Whole-cell extracts were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and examined using specific Abs to phosphorylated STAT1α (p-Tyr701; upper panel). The membrane was stripped and reprobed with Abs specific to unphosphorylated STAT1α to demonstrate equal protein loading (lower panel). Data are representative of at least two separate experiments. B, BAECs were pretreated with medium alone or Tkip (8 or 1 μM) overnight before 2-h incubation in serum-free medium. Following starvation, BAECs were treated with or without 50 ng/ml VEGF for 15 min in the presence or absence of various concentrations of Tkip (8 or 1 μM). VEGFR phosphorylation was monitored using immunoprecipitation (anti-VEGFR) and immunoblot (anti-pY) analysis. The membrane was stripped and reprobed with Abs specific to VEGFR to demonstrate equal protein loading (lower panel). Data are representative of at least two separate experiments.
effects of EMCV (Fig. 6). A lipophilic control peptide failed to affect IFN-γ antiviral activity, so the LT effect was due to Tkip. Thus, consistent with inhibition of JAK2 tyrosine kinase activity, Tkip blocked the antiviral effects of IFN-γ.

Another well-established function of IFN-γ is the up-regulation of MHC class I molecules on cells. Accordingly, we treated human WISH cells with 5000 U/ml human IFN-γ, which resulted in ≥3-fold increase in MHC class I as per FACS analysis (Fig. 7). Lipo-Tkip at 10 or 25 μM completely blocked up-regulation of MHC class I expression, and in fact reduced the baseline constitutive expression of MHC class I molecules. A control lipophilic peptide (IFN-γ95–125) had no effect. Thus, in addition to inhibition of IFN-γ antiviral activity, Tkip also inhibited IFN-γ up-regulation of MHC class I molecules. The fact that Tkip inhibited murine IFN-γ antiviral activity on WEHI cells (Fig. 6) and human IFN-γ up-regulation of MHC class I molecules on human WISH cells (Fig. 7) is consistent with the autophosphorylation sites of murine and human JAK2 (Table I) consisting of the same amino acid sequence.

Discussion
The JAK kinases play a central role in regulation of the biological activity of hormones and cytokines. JAK1 and JAK2 are the key tyrosine autophosphorylation kinases for mediation of signal transduction of IFN-γ (5). We have presented data in this study on the development of a 12-residue peptide, Tkip, which binds to the autophosphorylation site of JAK2. Tkip blocks both JAK2 autophosphorylation as well as phosphorylation of IFN-γ receptor subunit IFNγR-1. Consistent with binding to the autophosphorylation site of JAK2 and inhibition of autophosphorylation as well as IFNγR-1 tyrosine phosphorylation, Tkip also blocked both the antiviral activity and up-regulation of MHC class I molecules on cells treated with IFN-γ. Tkip inhibition of biological activity of IFN-γ was not associated with toxicity of the cells. By binding to the tyrosine autophosphorylation site of JAK2, Tkip represents a novel approach to specific control of tyrosine kinases. This has potential implications for regulation of inflammatory conditions and cancer where unregulated tyrosine kinases play a central role in the resultant pathology.

The fact that Tkip was developed to recognize the autophosphorylation site of JAK2, Tkip represents a novel approach to specific control of tyrosine kinases. Currently, there are eight identified members of the SOCS family, SOCS-1 to -7 and CIS. SOCS-1 and -3 are of interest here, because they are the negative regulators of JAK2 as well as several other cytokine and hormone receptor systems including EGFR (6–9, 19). In this regard, Tkip inhibited EGFR autophosphorylation, but VEGFR and c-src tyrosine kinases, which are not regulated by SOCS-1 and -3, were not affected by Tkip (21). Consistent with this, the VEGFR autophosphorylation site did not bind Tkip.

We initially showed in this study that Tkip bound to JAK2 autophosphorylation site where 1007Y of that site was not phosphorylated. This differs from SOCS-1 recognition of this site, where such binding occurs only when 1007Y is phosphorylated (13). However, although Tkip does bind to JAK2 autophosphorylation site in its nonphosphorylation state, we showed in this study that binding increased significantly when 1007Y was phosphorylated, indicating that Tkip has stronger affinity for the phosphorylated 1007Y at the JAK2 autophosphorylation site. Thus, there appear to...
be both similarities and differences in how Tkip and SOCS-1 recognize the JAK2 autophosphorylation site.

SOCS play an essential physiological role in maintenance of homeostasis. SOCS-1−/− mice, for example, develop normally through embryogenesis but die within 3 wk of a syndrome characterized by severe lymphopenia, fatty degeneration, necrosis of liver cells, and extensive macrophage infiltration of internal organs (22, 23). Similarly, SOCS-3−/− mice initially develop normally, but soon show marked liver erythropoiesis with resultant embryonic death (24, 25). The above defects appear to occur primarily as a result of unregulated IFN-γ and erythropoietin signaling. The persistent IFN-γ activity in the SOCS-1−/− mice is associated with abnormally activated T cells (reviewed in Ref. 8). The unregulated presence of IFN-γ results in extensive apoptosis. Evidence for the role of IFN-γ in the above condition is demonstrated by injecting SOCS-1−/− mice with Abs to IFN-γ, which results in abolishment of premature death, and a more normal appearance of internal organs (9). It would thus be of particular interest to determine whether Tkip can protect mice against the SOCS-1−/− lethal mutation. Both SOCS-1 and -3 are involved in regulation of JAK2, and the fact that both SOCS are needed would suggest that they act cooperatively in regulating overall JAK2 activity. SOCS-3 binds to the erythropoietin receptor with a KD of −1–10 μM (26). This is similar to the effective concentrations of Tkip for inhibiting IFN-γ antiviral and MHC class I up-regulation functions shown in this study. These data would suggest that we have shown proof-of-concept in the first reported development of a SOCS mimetic.

Given the primary function of SOCS-1 to negatively regulate signaling pathways mediated by cytokine binding, it is thus not surprising that SOCS-1 and other SOCS proteins may play key therapeutic roles in a variety of diseases associated with uncontrolled cytokine signaling (27). An example of the use of SOCS-1 as a potential therapeutic was demonstrated in the TEL-JAK2 model system (28–33). TEL-JAK2, an oncogene associated with human leukemia, arises from a chromosomal translocation leading to the fusion of the C-terminal segment of the JAK2 gene and the N-terminal segment of the TEL gene. The resulting fusion protein known as the TEL-JAK2 fusion protein contains the necessary domain required for JAK2 autophosphorylation (JH1) and thereby renders JAK2 constitutively active in cells leading to malignant cell proliferation. In the hemopoietic cell line Ba/F3, SOCS-1 was shown to act as a potent tumor suppressor and shown to effectively inhibit the kinase activity of the TEL-JAK2 fusion protein in vitro (28–33). It would thus be of great interest to investigate the effect of Tkip on hemopoietic cells constitutively expressing the TEL-JAK2 tyrosine kinase fusion protein.

Finally, the regions of SOCS-1 that are thought to bind to the autophosphorylation site of JAK2 involve a small domain, called the kinase inhibitory region (KIR), and a segment of a SH2 domain adjacent to KIR (13). The sequences of Tkip and KIR are compared for homology in Fig. 8. Sequence identity is seen with two F and R residues. Mutation analysis of the KIR of SOCS-1 have previously shown that F56 and F59 were critical for KIR binding to JAK2, with F59 being the most important residue (13, 27). These residues are conserved in both Tkip and SOCS-1. Future studies using amino acid substitution should better define Tkip specificity for JAK2 as well as the relationship of Tkip regulation to that of SOCS-1.

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