Structural Basis for c-KIT Inhibition by the Suppressor of Cytokine Signaling 6 (SOCS6) Ubiquitin Ligase*

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The c-KIT receptor tyrosine kinase mediates the cellular response to stem cell factor (SCF). Whereas c-KIT activity is important for the proliferation of hematopoietic cells, melanocytes and germ cells, uncontrolled c-KIT activity contributes to the growth of diverse human tumors. Suppressor of cytokine signaling 6 (SOCS6) is a member of the SOCS family of E3 ubiquitin ligases that can interact with c-KIT and suppress c-KIT-dependent pathways. Here, we analyzed the molecular mechanisms that determine SOCS6 substrate recognition. Our results show that the SH2 domain of SOCS6 is essential for its interaction with c-KIT pY568. The 1.45-Å crystal structure of SOCS6 SH2 domain bound to the c-KIT substrate peptide (c-KIT residues 564–574) revealed a highly complementary and specific interface giving rise to a high affinity interaction (Kd = 0.3 μM). Interestingly, the SH2 binding pocket extends to substrate residue position pY+6 and envelopes the c-KIT phosphopeptide with a large BG loop insertion that contributes significantly to substrate interaction. We demonstrate that SOCS6 has ubiquitin ligase activity toward c-KIT and regulates c-KIT protein turnover in cells. Our data support a role of SOCS6 as a feedback inhibitor of SCF-dependent signaling and provides molecular data to account for target specificity within the SOCS family of ubiquitin ligases.

Tyrosine kinase signaling from the stem cell factor (SCF)6 receptor, c-KIT is essential for the proliferation of primitive hematopoietic cells, melanocytes, and germ cells, but must be tightly regulated to avert the onset of multiple human tumors (1, 2). An autoinhibited state is maintained by the juxtamembrane (JM) region, which inserts between the N- and C-terminal kinase lobes blocking the activation loop (3). In response to SCF-induced receptor dimerization this lock is broken by phosphorylation of JM residues Tyr-568 and Tyr-570 and autophosphorylation of the kinase activation loop can proceed (4, 5). The JM region also contributes to downstream signaling by the docking of the SH2 proteins APS, SRC, SHP2, and c-CBL to phosphorytrosine 568 (pY568), and SHP1 to pY570 (6).

Cell proliferation induced by activated protein tyrosine kinases such as KIT, v-ABL, and TEL-JAK2 can be suppressed by feedback inhibitors, including suppressor of cytokine signaling (SOCS) family members, most notably SOCS1 (7–9). Tumor suppressor function is also proposed for SOCS3 and SOCS2 (10) and recently SOCS6 (11). The latter is down-regulated in a variety of cancers and has capacity to inhibit tumorigenesis when expressed in cell lines derived from gastric cancer (AGS and AZ-521) as well as non-small-cell lung cancer (H1299) and kidney (HEK293) (11). SOCS6 can bind directly to the JM region of c-KIT following SCF stimulation and phosphorylation of murine c-KIT Tyr-567 (human Tyr-568) (12). Consistent with this interaction, the ectopic expression of SOCS6 in a Ba/F3-KIT cell line caused a 40% decrease in SCF-dependent cell proliferation and a similar reduction in signaling through ERK1, ERK2, and p38 (12).

SOCS family proteins (SOCS1–7, CISH) share a conserved domain architecture, comprising a variable N terminus, a central SH2 domain, and a C-terminal SOCS box. A number of mechanisms have been described for inhibitory SOCS actions. Interactions mediated by the N-terminal or SH2 domains can provide kinase inhibitory activity or competitively inhibit substrate binding by other SH2-containing adapter proteins lead-
ing to attenuation of signaling (13). The SOCS box can also assemble into elongin B/C-containing E3 ligases and mediate degradation of substrate proteins (14). This has been demonstrated for SOCS6, which negatively regulates T cell activation by promoting proteasomal degradation of p56LCK in vivo (15). Interestingly, in this case the SH2 domain of SOCS6 was dispensable for the interaction with active LCK, which was instead mediated by a distal region in the N-terminal domain that contains no discernible phosphoprotein binding domain (15).

To further define the mechanisms underlying SOCS6 regulation of c-KIT and to confirm the interaction of the human proteins, we analyzed the contribution of different SOCS6 domains to its binding and inhibitory activity. In addition, we determined the crystal structure and affinity of the SOCS6/c-KIT substrate complex in vitro. Our analysis shows that the SOCS6 SH2 domain is essential for the interaction with c-KIT, while the SOCS box interaction with elongin B/C contributes to SOCS6 stability. Moreover, we demonstrate that SOCS6 is an E3 ubiquitin ligase for c-KIT in vitro and modulates its stability in vivo. The structure of SOCS6 reveals a highly complementary and specific interface for the bound c-KIT ligand. The determined high affinity is explained by the extension of the SH2 pocket to the pY+6 position and an elongated BG loop, which encloses the peptide adding significantly to the substrate interaction. Together our findings support the proposed role for SOCS6 as a feedback inhibitor of SCF-dependent signaling.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—To construct the pFlag-SOCS6 plasmid, DNA fragments encoding human SOCS6 (Uniprot ID: O14544) were amplified by PCR using Phu DNA polymerase (Fermentas, Burlington, Canada). The following primers were used: CATGAAATTCTAAGAAAATTAGTCTTAAAACCTT and CAGGATCTCTAGTATGCTTCTCCTGTGA. The restriction sites used for cloning are underlined. The PCR fragment was then inserted into the BamHI and EcoRI sites of the pFlag-CMV2 vector (Sigma-Aldrich). The site-directed mutagenesis used to create the R409E and C504F mutants was carried out with the QuikChange site-directed mutagenesis kit according to the manufacturer’s protocol. Insert and mutant sequences were confirmed by DNA sequencing.

**Cell Culture and Plasmid Transfection**—HEK293T were cultured in Dulbecco’s modified Eagle’s medium medium containing 10% fetal bovine serum (FBS), l-glutamine, penicillin, streptomycin (Invitrogen). DNA plasmid transfections were carried out with the manufacturer’s instructions on 70–80% confluent cells using Superfect reagent (Qiagen). Cells were serum-starved overnight 24 h after transfection in 0.5% FBS medium and then stimulated for 7 min with 150 ng/ml human SCF (ProSpec, Tany, Rehovot, Israel).

**Antibodies**—Monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology. Anti-β-actin, anti-c-Myc, anti-elongin B, and IgG horseradish peroxidase antibodies were from Santa Cruz Biotechnology. Anti-hemagglutinin and anti-Flag (M2) antibodies were purchased from Sigma-Aldrich. Anti-KIT antibody has been described previously (17).

**Immunoprecipitation and Western Blot**—To immunoprecipitate c-KIT, clarified whole cell lysates (WCL) were mixed for 18 h with 1 μg of antibody and a bed volume of 10 μl of protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden). The immunoprecipitated (IPs) were washed three times with lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 1× mixture inhibitor (Roche, Penzberg, Germany), 20 mM NaF, 1 mM PMSF, 5 μM MG132) and dissolved in SDS sample buffer. Following SDS-PAGE, proteins were transferred to membranes (Immobilon-P, Millipore) and probed with different antibodies.

**In Vitro E3 Ubiquitin Ligase Activity Assays**—To determine SOCS6 E3 ligase activity, HEK293T cells were transfected with pFlag-SOCS6 and elongins B and C in 10-cm plates. After SCF stimulation, cells were homogenized in lysis buffer A. Clarified whole cell lysates were mixed with 1 μl of anti-Flag-agarose beads (Sigma-Aldrich) for 1 h. The immunoprecipitates (IPs) were washed with lysis buffer followed by three washes with HEB buffer (20 mM Tris-HCl pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM DTT, and 10 μM MG132). The equilibrated IPs were then incubated for 30 min at 30 °C in 100 μl of HEB buffer containing 1 μg of E1 (human recombinant His-tagged, Enzo Life Sciences, Inc.), 1.5 μg of E2 (UbcH5b, Enzo, Life Sciences, Inc.), 16 μM ubiquitin, 5 μM HA-ubiquitin, 0.8 mM ATP, 0.8 mM MgCl2, 25 mM creatine phosphate, and 0.03 ng of rabbit skeletal muscle creatine kinase (Calbiochem, La Jolla, CA). Aliquots of the reactions were then stopped by the addition of electrophoresis sample buffer, followed by SDS-PAGE and immunoblotting. In a parallel set of experiments, the in vitro ubiquitination reactions were stopped by addition of EDTA (20 mM final concentration), followed by elution in HEB buffer containing 100 μg/ml Flag peptide (Sigma-Aldrich) for 1 h at 4 °C. Samples containing the soluble SOCS6-containing complexes were recovered after centrifugation (500g, 2 min) followed by overnight c-KIT immunoprecipitation using 1 μg of antibody per sample.

**In Vivo c-KIT Ubiquitination**—HEK293T were transfected with elongin B/C and HA-ubiquitin. After overnight starvation with 0.5% FBS, cells were treated with 150 ng/ml SCF for 10 min. After SCF stimulation, cells were lysed with lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 10% glycerol, 1.5 mM MgCl2, 1 mM Na3VO4, 1× mixture inhibitor, 50 mM NaF, 1 mM PMSF, and phosphatase inhibitor mixture 2 (Sigma-Aldrich), 10 μM MG132). Aliquots of whole cell lysates were subjected to immunoprecipitation of c-KIT followed by Western blot for HA-tagged ubiquitin.

**Cycloheximide Chase Experiments**—HEK293T cells were transfected with pFlag-SOCS6 and elongin B/C. After 24 h post-transfection, cells were split into different plates for different treatments. Cells were treated with 100 μg/ml cycloheximide (Sigma-Aldrich) for 30 min and then lysed at different time points.
Structural Basis for c-KIT Inhibition by SOCS6

Protein Expression—The ESS and SH2 domains of human SOCS6 (amino acids 361–499) were cloned into the vector pNIC-Bsa4 by ligation-independent cloning (LIC). DNA sequencing identified two amino acids changes (V368D, R463G) most likely introduced by PCR error; both positions were solvent exposed in the SOCS6 structure. Protein was expressed at 18 °C in BL21 (DE3) cells. Harvested cells were buffered in 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 5 mM imidazole, 1 mM PMSF, 0.5 mM TCEP, and lysed by sonication. An initial purification was performed using nickel affinity and size exclusion chromatography on a HiLoad 16/60 Superdex 75 column. For a final clean up step SOCS6 protein was passed through a Source15Q anion-exchange column buffered in 50 mM HEPES pH 7.5 and collected in the flow-through. The N-terminal hexahistidine tag was cleaved using TEV protease and the expected molecular weight confirmed by LC-ESI mass spectrometry. The buffer was adjusted to 25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM DTT, 50 mM L-arginine, 50 mM L-glutamate for crystallization.

Isothermal Titration Calorimetry—Experiments were performed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT at 20 °C, injecting 250 μM peptide solution into 20 μM protein solution. Blank titrations were subtracted from binding data, and data were processed using ORIGIN software provided with the instrument. The pY568 peptide comprised human c-KIT residues 564–574 (NGNNpYVYIDPT) and was synthesized by ThermoScientific. The pY936 peptide comprised human c-KIT residues 932–942 (TNHIPYSNLANC) and was synthesized by Genescript.

SPOT Peptide Array Synthesis and Probing—The SPOT peptide array representing phosphoryrosine sites in the c-KIT and PDGFR receptor tyrosine kinases was synthesized on a functionalized cellulose membrane using an Auto-Spot ASP 222 Robot (Abimed). The SPOT membrane was blocked with 5% skim milk in TBST (0.1 M Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h. Purified recombinant GST-SOCS6-SH2 or GST was added directly in the blocking buffer to a final concentration of 1 μg/ml protein and incubated with the membrane at room temperature for 1 h. The membrane was then washed 3 × 5 min with TBST before a rabbit anti-GST antibody (Santa Cruz Biotechnology) was added. The membrane was allowed to incubate at room temperature for 30 min prior to 3 × 5-min washes with TBST. A goat anti-rabbit GST-horseradish peroxidase conjugate was then added and incubated with the membrane for another 30 min. After final 3 × 5-min washes in TBST, the SPOT membrane was visualized by enhanced chemiluminescence and quantified by densitometry.

Crystallization—SOCS6 protein was concentrated to 13.6 mg/ml in the presence of a molar excess of c-KIT pY568 peptide (NGNNpYVYIDPT). A further 2 mM peptide was added to the final sample. Crystals were grown from sitting drops comprising 50 nl of SOCS6-peptide complex and 100 nl of reservoir solution (0.2 M ammonium sulfate, 30% (w/v) PEG 5000 monomethylether, 0.1 M MES pH 6.5) at 4 °C. Large plates appeared within 3 days. Crystals were transferred briefly to reservoir solution supplemented with 20% (v/v) ethylene glycol prior to vitrification in liquid nitrogen.

Data Collection—Data were collected from a single crystal at 100 K on beamline X10SA at the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). Data were recorded to a nominal resolution of 1.4 Å using a MAR225 CCD detector. Data were indexed, integrated, and reduced using MOSFLM (18) and SCALA (19). The crystals were orthorhombic and based on unit cell volume and symmetry, each asymmetric unit was predicted to contain a single SOCS6-peptide complex giving rise to a Matthews coefficient of 2.04 and a solvent content of 40%.

Phasing, Model Building, Refinement, Validation—Initial phase estimates were obtained by molecular replacement using the program PHASER (20) with the coordinates of SOCS4 SH2 domain (PDB: 2IZV) as a search model. Initial electron density maps clearly showed the presence of the c-KIT phosphopeptide. Model building was carried out manually with COOT (21) interspersed with cycles of restrained refinement with REFMAC5 (5.3.0040) (22) using TLS (translation-libration-screw) and isotropic B-factor refinement. In the final stages, full anisotropic temperature factor refinement was used. All data to 1.4 Å were used for refinement apart from the final cycle where the resolution was truncated to 1.45 Å (the quoted resolution of the structure based on data completeness according to in-house criteria). Data collection and refinement statistics are shown in Table 1.

The final model comprises residues Val-361 to Ser-363 and Ser-317 to Gly-496 of SOCS6, the Asn-566 to Thr-574 of the c-KIT pY568 phosphopeptide, a single ethylene glycol, and 129 water molecules. SOCS6 residues Ser-364—Thr-370 and three residues at the C terminus (497–499) have poorly defined electron density and have not been included in the model. The final model has excellent stereochemistry and geometry as adjudged by the MOLPROBITY server. The figures were prepared using ICM-PRO (23).

RESULTS

The SH2 Domain of SOCS6 Mediates the Interaction with c-KIT—The role of SOCS6 as a negative regulator of SCF/c-KIT signaling has been previously demonstrated (12). To better characterize the molecular basis for these actions, we generated Flag-tagged SOCS6 variants with point mutations disrupting protein–protein interactions in the SOCS box (C504F) and in the SH2 domain (R409E) and expressed them in HEK293T, a cell line that endogenously expresses the c-KIT tyrosine kinase receptor. The conserved SOCS6 residue Cys-504 is located within the SOCS box, and its mutation to phenylalanine disrupts elongin C interaction (24). SOCS6 Arg-409 is a conserved residue within the phosphotyrosine binding SH2 domain and contributes significantly to phosphotyrosine binding (25). As shown in Fig. 1A, co-expression of SOCS6 with elongin C and B resulted in the stable expression of SOCS6. In contrast, cells transfected with a SOCS6-C504F expression vector failed to express the SOCS6 variant at detectable levels. The expression of SOCS6-R409E was also reduced relative to wild type (WT). Given the dramatic effect of the C504F mutation, which lies in the interface between the SOCS box and elongin C (25, 26), we tested the influence of elongin C and B expression on the levels of SOCS6. Co-
expression of elongin C and B with SOCS6 resulted in significantly higher expression of both SOCS6-WT and SOCS6-R409E, indicating that the interactions with elongin C/B contributed to the stability of exogenously expressed SOCS6. To further substantiate this finding, we used cycloheximide (CHX) chase experiments to measure SOCS6 protein half-life (Fig. 1B) and found that co-expression of SOCS6 and elongin B resulted in significant reduction of SOCS6 turnover, confirming the elongin contribution to SOCS6 stability. Expression levels of SOCS6 were analyzed by Western blot.

SOCS6 Promotes Ubiquitination of c-KIT Receptor—We next analyzed the contribution of the SH2 domain to the interaction with c-KIT. As shown in Fig. 2A, the binding of c-KIT to SOCS6-WT was confirmed by co-immunoprecipitation and was significantly enhanced upon SCF stimulation concomitant with increased tyrosine phosphorylation of the c-KIT receptor. In contrast, no association was observed between c-KIT and the SOCS6-R409E variant, despite the fact that similar amounts of SOCS6 and SOCS6-R409E were immunoprecipitated. The SOCS6-R409E protein also showed a significantly reduced expression level concomitant to a diminished association with elongin C/B (perhaps reflecting some local structural perturbation). Thereby, confirming that interactions with elongins C/B contribute to the stability of SOCS6.

Interestingly, the SOCS6-R409E protein retained its ability to interact with cullin 5, which is known to bind SOCS family members through a motif located C-terminal to the elongin C-interacting motif (27) and functions to link SOCS with the E2 ubiquitin conjugation system. Consequently, cullin5 is essential for the activity of the Elongin/Cullin/SOCS (ECS) family of ubiquitin ligases.

Subsequently, we analyzed whether SOCS6 inhibitory actions on c-KIT signaling (12) are due to its function as an E3 ubiquitin ligase. To explore this hypothesis we first tested SOCS6 activity using an in vitro ubiquitination assay: Flag-tagged SOCS6 as well as the SOCS6-R409E mutant were expressed in HEK293T cells, immunoprecipitated, and mixed with recombinant E1, the ubiquitin-conjugating enzyme, UbcH7, HA-tagged ubiquitin and an ATP-regenerating system. Ubiquitin ligase activity was measured by the appearance of high molecular weight poly-HA-ubiquitin conjugates. As shown in Fig. 2B (middle panel), the inclusion of SOCS6 dramatically increased the ubiquitin ligase activity. This activity was dependent on the presence of the E1 and E2 enzymes as well as ATP. As expected, negative controls in the absence of HA-ubiquitin showed no signal. Having demonstrated SOCS6-associated ubiquitin ligase activity, we also addressed whether c-KIT was ubiquitinated by SOCS6 (Fig. 2B, upper panel). c-KIT was immunopurified from the in vitro ubiquitination reactions, and its ubiquitination status was probed by Western blotting with antibodies against HA-ubiquitin. The results clearly demonstrated dose-dependent ubiquitination of c-KIT in the presence of SOCS6. A control, using the SOCS6-R409E mutant confirmed the reduced ability of the SH2 mutant to associate with c-KIT and consequently a marked reduction in the ubiquitination of c-KIT by this SOCS6 variant. Interestingly, reduced ubiquitination of c-KIT by the SOCS6-R409E mutant cannot be attributed to reduced intrinsic E3 ligase activity of the mutant because SOCS6-R409E showed comparable activity to SOCS6-WT, in line with its capacity to associate with cullin5. This finding is consistent with the ability of SOCS6 to mediate ubiquitination of interacting proteins independently of its SH2 domain as demonstrated for the SOCS6 degradation of tyrosine kinase LCK (15).

Similar results were obtained in cellular model systems, where the expression of SOCS6 increased SCF-stimulated ubiquitination (Fig. 2C), although significant ubiquitination was also observed in control cells, probably through the actions of endogenous ubiquitin ligases. Critically, cells expressing SOCS6 also showed a reduced half-life of endogenous c-KIT as measured in CHX chase experiments (Fig. 2D).

Structure Determination of a High Affinity SOCS6/c-KIT Complex—Having shown the importance of the human SOCS6 SH2 domain to the interaction with c-KIT we proceeded to analyze this interface in detail. Isothermal titration calorimetry (ITC) measurements showed submicromolar binding of SOCS6 to a phosphopeptide derived from the c-KIT juxtamembrane region, pY568 (\(K_d = 0.3\) μM) (Fig. 3A). Interestingly, the binding was characterized by low enthalpy, but strongly favorable entropy, perhaps as a result of dehydration upon binding. No discernable interaction was observed for SOCS6 binding to a C-terminal site encompassing c-KIT pY936 (\(K_d > 10\) μM, data not shown). To understand the structural basis for the high affinity juxtamembrane interac-
tion, we crystallized the SOCS6 SH2 domain in complex with the c-KIT pY568 peptide. Crystals were obtained in space group P2₁2₁2₁ with one SOCS6-peptide complex per asymmetric unit. The determined structure, refined to 1.45 Å resolution, comprised the N-terminal extended SH2 subdomain (ESS residues 371–383) as well as the SH2 domain (residues 384–496). Crystal contacts allowed five additional N-terminal residues to be defined (two remaining from the hexahistidine tag and SOCS6 361–363). At the SH2 C terminus three linker residues were located that would connect to the SOCS box in the intact protein. The bound peptide (human c-KIT residues 564–574; NGNNpYVYIDPT) was extremely well defined in the electron density maps except for the two N-terminal positions (see Table 1 for data collection and refinement statistics).

Overview of the SOCS6 Structure—Overall, SOCS6 adopts the canonical SH2 fold (Fig. 3, B and C). The accompanying N-terminal ESS helix is, however, an unique feature of the
SOCS family packing below the BC and DE loops. Although annotated originally only in the SOCS1–3/CISH subfamily, secondary structure predictions suggest that this motif is present also in SOCS4–7, and this is now confirmed experimentally in the SOCS4 (26) and SOCS6 structures, although these helices are a turn shorter than in SOCS2 (25) and SOCS3 (28, 29). Consistent with these previous structures, the SOCS6 ESS is amphipathic and uses the hydrophobic surface (Leu-372/Leu-376/Leu-379) to bind and stabilize the SH2 domain. An additional hydrogen bond is formed between Glu-375 (ESS) and the backbone amide of Ile-454 in the αB helix. High structural conservation is also observed in the N-terminal half of the SH2 domain, which comprises the phosphotyrosine binding pocket (including αA to βD). The C-terminal SH2 region is more variable and in SOCS6 is distinguished by a greatly extended BG loop and a relatively long αB helix.

Specific Interactions of the c-KIT Phosphopeptide—The structure of the complex reveals an extended peptide conformation as typical of other SH2 phosphopeptide interactions (Fig. 4A). In previous SOCS3 structures the phosphate moiety of the phosphotyrosine was inverted compared with typical SH2s like SRC (28, 29). Consistent with these previous structures, the SOCS6 ESS is amphipathic and uses the hydrophobic surface (Leu-372/Leu-376/Leu-379) to bind and stabilize the SH2 domain. An additional hydrogen bond is formed between Glu-375 (ESS) and the backbone amide of Ile-454 in the αB helix. High structural conservation is also observed in the N-terminal half of the SH2 domain, which comprises the phosphotyrosine binding pocket (including αA to βD). The C-terminal SH2 region is more variable and in SOCS6 is distinguished by a greatly extended BG loop and a relatively long αB helix.

**FIGURE 3.** Overall structure and affinity of the SOCS6/c-KIT peptide complex. A, ITC measurement of the c-KIT pY568 peptide binding to the SOCS6 SH2 domain. SOCS6 binds with $K_D = 0.3 \mu M$ ($K_S = 32.3 \pm 4.2 \times 10^5 M^{-1}$, $\Delta H^\text{obs} = -229 \pm 0.03 \text{ kcal/mol}, \Delta S = 6.45 \text{ kcal/mol}, \Delta G = -8.74 \text{ kcal/mol}, n = 1.02$). A blank titration is colored blue and offset for clarity. B, ribbon diagram of the structure of the SOCS6/c-KIT complex. The ESS helix and SH2 domain are colored blue and orange, respectively. Part of the C-terminal linker to the SOCS box is colored red. Selected secondary structure elements are labeled. The c-KIT peptide is shown in stick representation and colored green. C, same view showing a surface representation of SOCS6 colored by electrostatic surface potential between −10 and +10 kcal/electron units.

**TABLE 1**

| Data collection and refinement statistics | SOCS6 + peptide |
|----------------------------------------|-----------------|
| PDB code                               | V2IF            |
| Ligand                                 | c-KIT pY568 peptide (564–574) |
| Space group                            | P2, 1_2, 1_2    |
| Cell dimensions (Å)                    | $a = 30.38, b = 60.14, c = 71.37$ |
| a, b, g                                | 90, 90, 90      |
| Crystallization conditions             | 0.2 M ammonium sulphate, 30% (w/v) |
| Data collection                        | Resolution (Å)$^a$ 1.40 (1.40–1.48) |
|                                       | Unique observations 26242 |
|                                       |Completeness% [%] 98.8 (91.5) |
|                                       |Redundancy% [%] 4.3 (2.7) |
|                                       |$R_{	ext{merge}}$$^b$ 0.091 (0.868) |
|                                       |$R_{	ext{free}}$ 0.041 (0.619) |
|                                       |I/s$^b$ 12.4 (2.0) |
| Refinement                             | Resolution (Å) 35.69–1.45 |
|                                       |Reflections ($R_{	ext{meas}}$) 22652 (1210) |
|                                       |$R_{	ext{merge}}$/R$_{\text{free}}$% 13.9/19.8 |
|                                       |Atoms (P/L/W/O)$^b$ 1091/81/129/4 |
|                                       |B-factors (P/L/W/O)$^b$/Å$^2$ 17/19/31.6/20.2 |
|                                       |r.m.s.d bonds (Å) 0.013 |
|                                       |r.m.s.d angles [°] 1.475 |
|Ramachandran$^a$                       |Favored [%] 98.6 |
|                                       |Allowed [%] 1.4 |

$^a$Values in brackets represent statistics for highest resolution shells.

$^b$(P/L/W/O): protein atoms, ligand atoms, water, other.

$^c$Molprobity server analysis (molprobity.biochem.duke.edu/).
Previous studies have determined SOCS6 preferences for valine, tyrosine, and isoleucine at positions pY+1 to pY+3 relative to the phosphotyrosine, matching precisely those residues found in the c-KIT ligand (30). The specificity of SOCS6 for these c-KIT residues is explained by their enclosure within a hydrophobic channel formed by the EF and BG loops (Figs. 4C and 3C). The close contact of the pY+1 residue Val-569 with SOCS6 Phe-422, Leu-429, and Tyr-472 would disfavor the binding of larger aromatic or charged residues. The aromatic side chain of the pY+2 residue Tyr-570 stacks between SOCS6 Arg-476 and Tyr-443 and makes additional van der Waals contacts with the EF and BG loops. The SOCS6 Arg-476 side chain rests above the peptide and extends also to hydrogen bond with the backbone carbonyl of the pY+3 residue Ile-571. Here, the isoleucine side chain buries into the SH2 core formed by SOCS6 Phe-422, Phe-442, and Phe-470 (Fig. 4C).

A key feature of the SOCS6/c-KIT complex is the extensive hydrogen bonding with the substrate (Figs. 4 and 5). Overall, hydrogen bonds are formed with the c-KIT main chain from the pY-2 to pY+4 positions, and with the c-KIT side chains from the pY-1 to pY+6 positions (Fig. 5). SH2 contact with the pY+6 residue is unusual and involves two side-chain hydrogen bonds from SOCS6 H460 and D464 (aB) to the OG1 oxygen of c-KIT T574 (Fig. 4D). Other notable interactions are formed with the c-KIT main chain. Most significantly, a mini three-stranded antiparallel β-sheet is formed between c-KIT and the extended BG loop (strands βBG1 and βBG2) (Fig. 4D). Here, the pY+2 and pY+4 main-chain atoms are bound by the main chain of SOCS6 Ala-469, Cys-471 and Ser-473, although water-mediated hydrogen bonds bridge to the pY+4 carbonyl. The intervening pY+3 carbonyl additionally receives side-chain hydrogen bonds from both the EF (Gln-445) and BG (Arg-476) loops. A further water-mediated hydrogen bond to the pY+3 amide is split between the EF loop (Tyr-443 carbonyl) and the βD5 side chain Thr-431, which also binds directly to the pY+1 carbonyl. Finally, canonical SH2 interactions are made by the main chain of the central βD4 residue His-340 with the pY+1 amide and pY-1 carbonyl (Figs. 4C and 5).

Comparison with the SOCS3 and SHP2 SH2 Domains—The extended interface of the SOCS6/c-KIT complex establishes a buried surface area (1714 Å²) similar to that observed in the SOCS3/gp130 complex (1761 Å²) and far greater than for most other SH2 domains (28, 29). These complexes provide the first SOCS-peptide structures available for comparison and reveal striking differences in the interactions of the EF and BG loops that explain their different activities (Fig. 6A). In common with SOCS6, the SOCS3 SH2 domain contains an extended BG loop that contacts the substrate backbone through the βBG1-βBG2 hairpin. However, in contrast to SOCS6, further specific contacts in the SOCS3 complex are formed almost entirely by the opposite EF loop, which is four residues longer in SOCS3. As a result SOCS3 derives specificity for gp130 through a deep pocket that accommodates the pY+5 residue His-762 (28, 29).

Proteomic analyzes of SH2 specificity place the SOCS family with the phosphatase SHP2 (PTPN11) in a subset of SH2
domains that recognize the consensus motif pYΦXΦ, where Φ represents any hydrophobic amino acid (31). SHP2 also targets the pY568 site in c-KIT. Moreover, the structure of the N-terminal SH2 domain of SHP2 is strikingly similar to SOCS6 (Fig. 6). In particular, the EF and BG loop conformations are closely matched, although these secondary structure elements are shorter in the SHP2 domain, perhaps reflecting its more promiscuous activity. In agreement, the main-chain conformation of the c-KIT peptide closely resembles that of the PDGFR pY1009 peptide bound to SHP2 (PDB 1AYA), which also contains a pY+5 proline residue (Fig. 6C).
These structural comparisons bring in to question whether PDGFR is also targeted by SOCS6. PDGFR and c-KIT are closely-related kinases and show high sequence similarity, particularly in their juxtamembrane domains. To test this hypothesis, we prepared a SPOT peptide array of phosphotyrosine sites in PDGFR and c-KIT and probed the array with SOCS6 or control GST protein. As shown in Fig. 6D, SOCS6 bound c-KIT specifically at pY568, with additional weaker binding to the doubly phosphorylated site pY568/pY570. SOCS6 showed no binding to PDGFR pY1009, which lacks critical hydrophobic contacts such as the pY/H11001 tyrosine. SOCS6 binding to PDGFR pY579 in the juxtamembrane region was also only weakly detected. This site has close conservation of c-KIT residues pY/H11001 and pY/H11006. Together, these results highlight the specificity of the crystallized SOCS6/c-KIT complex.

**Domain Organization in the SOCS C Terminus**—The ESS and BG loop insertions present in SOCS family members have hindered sequence alignments and correct assignments of the SOCS SH2 domain boundary for expression studies (27). The determined SOCS structures identify a consensus LXXPL/V motif in the C-terminal βG strand that is conserved in other SH2 families (Fig. 7A). The aliphatic leucine residues straddle the βG strand to make important contributions to the SH2 core (Fig. 7A). Further downstream residues in the SOCS family form a linker (βG-H1 loop) to the C-terminal SOCS box domain (Fig. 7B). Structural alignment of the βG strand shows that the linker length is variable and longest in SOCS6 (Fig. 7C). The linker forms a spacer that allows the SOCS box of the SOCS1–3/CISH and SOCS4–7 subfamilies to extend around the ESS helix and pack on opposite faces of the SH2 domain (26). Comparison of the SOCS4/elongin C/B and SOCS6 structures shows that the SOCS6 ESS helix extends one additional turn before the SH2 domain (Fig. 7B). Therefore, the linker insertion in SOCS6 is most likely required to negotiate this additional ESS turn.

**DISCUSSION**

We have demonstrated that SOCS6, via its SH2 domain, interacts with c-KIT to promote its ubiquitination and regulates c-KIT receptor turnover in vivo. The interaction site in the juxtamembrane region, c-KIT pY568, binds with high affinity (Kd = 0.3 μM) and conforms to the preferred recognition sequence determined for SOCS6 (pYVYI) (30). The observed specificity can be explained by a BG loop insertion in the SH2 domain of SOCS6 which envelopes the c-KIT peptide to provide extensive hydrogen bonding as well as hydrophobic interaction. The SH2 loop arrangement is similar to SHP2, which also binds c-KIT pY568, but displays striking differences to other SOCS family members, including SOCS3, that target other receptors.

All SOCS family members are believed to share a common mechanism of action whereby the C-terminal SOCS box domain interacts with elongin C and B as well as cullin5/rbx2 to assemble a core multimeric complex with ubiquitin ligase.
activity (25). It has been shown that SOCS6 binds cullin5 with far greater affinity ($K_a = 23 \text{ nm}$) than SOCS1 ($K_a = 1 \text{ nm}$) or SOCS3 ($K_a = 0.1 \text{ nm}$), suggesting that ubiquitin ligase activity is the primary mechanism for SOCS6 inhibitory actions (32). The demonstration of this activity toward c-KIT and reduction in c-KIT protein levels in cellular systems, strongly suggests that SOCS6-mediated ubiquitination is important for its regulation of c-KIT receptor turnover. However, the high affinity of the SOCS6 SH2 domain also provides potential for the competitive inhibition of other signaling intermediaries to Tyr-568. The SOCS1 and SOCS3 proteins additionally contain a kinase inhibitory motif located N-terminal to the ESS domain that targets JAK2 catalytic activity (13, 33). Consequently, SOCS box deletion mutants of SOCS1 retain inhibitory activity against INFγ receptor signaling (34). The existence of a similar kinase inhibitory domain in SOCS6 remains to be demonstrated, but is not supported by our finding that SOCS6 expression does not inhibit the rapid phosphorylation of c-KIT induced by SCF. Moreover, the interaction is inhibited by mutation of c-KIT Tyr-568 (12) or by mutation of the SOCS6 SH2 domain.

Interestingly, a recent publication suggests that SOCS6 ubiquitin ligase activity can also modulate p56LCK stability through an interaction that involves the N-terminal but not the SH2 domain of SOCS6 (15). The N-terminal region is also necessary for SOCS6 negative regulation of STAT3 protein levels (35). Therefore, SOCS6 seems to contain two distinct substrate recognition motifs.

Phosphorylation of Tyr-568 and subsequently of Tyr-570 is an early and necessary event in the activation of c-KIT signaling, effecting the release of autoinhibitory interactions between the receptor juxtamembrane and kinase domains (4, 36). In gastrointestinal stromal tumors (GIST), a cluster of somatic mutations has been identified within the juxtamembrane region resulting in the constitutive activation of c-KIT (37, 38). Most of these mutations occur upstream of the Tyr-568 residue and none of them involve SOCS6 interacting residues. This is likely due to the fact that phosphorylation of Tyr-568 is needed to recruit a number of signaling intermediaries that mediate activation of the downstream signaling pathway. Of these molecules SRC kinase is of special interest as it is considered essential for MAP kinase activation upon SCF treatment, an event that is negatively regulated by SOCS6 (12, 40). Although we provide strong evidence for the existence of a high affinity interaction between c-KIT pY568 and SOCS6, we cannot exclude that SOCS6 also regulates c-KIT function indirectly, perhaps through downstream signaling intermediaries. Indeed, previous studies have demonstrated SOCS6 interactions with known downstream factors, including STAT3 and the p85 regulatory subunit of PI3 kinase (39, 41), suggesting that SOCS6 regulates SCF-dependent cellular proliferation on multiple levels. Additional studies are clearly needed to see whether these interactions are of functional relevance, whether they involve the SOCS6 SH2 domain and whether the phosphotyrosine residues responsible for the interaction conform to the features observed in the structure of SOCS6/c-KIT we have analyzed.

In summary, our data support the role of SOCS6 as an E3 ubiquitin ligase with activity toward c-KIT and describes the details of the target recognition by its SH2 domain. Comparative analyses based on our data can provide a molecular explanation for the target specificity exhibited by different SOCS family members; information that may lead to a better understanding of the distinct physiological functions of these proteins.

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REFERENCES
1. Hubbard, S. R. (2004) Nat. Rev. Mol. Cell. Biol. 5, 464–471
2. Dibb, N. J., Dilworth, S. M., and Mol, C. D. (2004) Nat. Rev. Cancer 4, 718–727
3. Mol, C. D., Dougan, D. R., Schneider, T. R., Skene, R. J., Kraus, M. L., Scheibe, D. N., Snell, G. P., Zou, H., Sang, B. C., and Wilson, K. P. (2004) J. Biol. Chem. 279, 31655–31663
4. Mol, C. D., Lim, K. B., Sridhar, V., Zou, H., Chien, E. Y., Sang, B. C., Nowakowski, J., Kassel, D. B., Cronin, C. N., and McMee, D. E. (2003) J. Biol. Chem. 278, 31461–31464
5. Yuzawa, S., Opatowsky, Y., Zhang, Z., Mandiyan, V., Lax, I., and Schlessinger, J. (2007) Cell 130, 323–334
6. Rönnstrand, L. (2004) Cell Mol. Life Sci. 61, 2535–2548
7. Kamizono, S., Hanada, T., Yasukawa, H., Minoguchi, S., Kato, R., Minoguchi, M., Hattori, K., Hatakeyama, S., Yada, M., Morita, S., Kitamura, T., Kato, H., Nakayama, K., and Yoshimura, A. (2001) J. Biol. Chem. 276, 12530–12538
8. Frantzve, I., Schwaller, J., Sternberg, D. W., Kukot, J., and Gilliland, D. G. (2001) Mol. Cell. Biol. 21, 3547–3557
9. Rottapel, R., Ilgunamuran, S., Neale, C., La Rose, J., Ho, M. J., Nguyen, M. H., Barber, D., Dubreuil, P., and de Sepulveda, P. (2002) Oncogene 21, 4351–4362
10. Hendriksen, P. J., Dits, N. F., Kokame, K., Veldhoven, A., van Weerden, W. M., Bangma, C. H., Trapman, J., and Jenster, G. (2006) Cancer Res. 66, 5012–5020
11. Lai, R. H., Hsiao, Y. W., Wang, M. J., Lin, H. Y., Wu, C. W., Chi, C. W., Li, A. T., Jou, Y. S., and Chen, J. Y. Cancer Lett. 288, 75–85
12. Bayle, J., Letard, S., Frank, R., Dubreuil, P., and De Sepulveda, P. (2004) J. Biol. Chem. 279, 12249–12259
13. Yasukawa, H., Misawa, H., Sakamoto, H., Sakuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N., and Yoshimura, A. (1999) EMBO J. 18, 1309–1320
14. Kile, B. T., Schulman, B. A., Alexander, W. S., Nicola, N. A., Martin, H. M., and Hilton, D. J. (2002) Trends Biochem. Sci. 27, 235–241
15. Choi, Y. B., Son, M., Park, M., Shin, J., and Yun, Y. (2010) J. Biol. Chem. 285, 7271–7280
16. Lerner, M., Corcoran, M., Cepeda, D., Nielsen, M. L., Zubarev, R., Pontén, F., Uhlén, M., Hober, S., Grandér, D., and Sangaleti, O. (2007) Mol. Biol. Cell 18, 1670–1682
17. Sun, J., Pedersen, M., and Rönnstrand, L. (2008) J. Biol. Chem. 283, 27444–27451
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18. Leslie, A. G. W. (2006) *Acta Crystallogr. D* **62**, 48–57
19. Evans, P. (2006) *Acta Crystallogr. D* **62**, 72–82
20. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) *J. Appl Crystallogr* **40**, 658–674
21. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) *Acta Crystallogr D* **66**, 486–501
22. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) *Acta Crystallogr D* **53**, 240–255
23. Abagyan, R. A., Totrov, M., and Kuznetsov, D. (1994) *J Comp Chem* **15**, 488–506
24. Kamura, T., Burian, D., Yan, Q., Schmidt, S. L., Lane, W. S., Querido, E., Branton, P. E., Shlattifard, A., Conaway, R. C., and Conaway, J. W. (2001) *J. Biol. Chem.* **276**, 29748–29753
25. Bullock, A., Debreczeni, J. E., Edwards, A. M., Sundström, M., and Knapp, S. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103**, 7637–7642
26. Bullock, A. N., Rodriguez, M. C., Debreczeni, J. E., Songyang, Z., and Knapp, S. (2007) *Structure* **15**, 1493–1504
27. Kamura, T., Maenaka, K., Kotoshiba, S., Matsumoto, M., Kohda, D., Conaway, R. C., Conaway, J. W., and Nakayama, K. I. (2004) *Genes Dev.* **18**, 3055–3065
28. Babon, J., McManus, E., Yao, S., DeSouza, D., Mielske, L., Sprigg, N., Willson, T., Hilton, D., Nicola, N., Baca, M., Nicholson, S., and Norton, R. (2006) *Mol. Cell* **22**, 205–216
29. Bergamin, E., Wu, J., and Hubbard, S. R. (2006) *Structure* **14**, 1285–1292
30. Krebs, D. L., Uren, R. T., Metcalf, D., Rakar, S., Zhang, J. G., Starr, R., De Souza, D. P., Hanzinikolas, K., Eyles, J., Connolly, L. M., Simpson, R. J., Nicola, N. A., Nicholson, S. E., Baca, M., Hilton, D. J., and Alexander, W. S. (2002) *Mol. Cell. Biol.* **22**, 4567–4578
31. Huang, H., Li, L., Wu, C., Schibli, D., Colwill, K., Ma, S., Li, C., Roy, P., Ho, K., Songyang, Z., Pawson, T., Gao, Y., and Li, S. S. (2008) *Mol. Cell. Proteomics* **7**, 768–784
32. Babon, J. J., Sabo, J. K., Zhang, J. G., Nicola, N. A., and Norton, R. S. (2009) *J. Mol. Biol.* **387**, 162–174
33. Nicholson, S., Willson, T., Farley, A., Starr, R., Zhang, J., Baca, M., Alexander, W., Metcalf, D., Hilton, D., and Nicola, N. (1999) *EMBO J.* **18**, 375–385
34. Zhang, J. G., Metcalf, D., Rakar, S., Asimakis, M., Greenhalgh, C. J., Willson, T. A., Starr, R., Nicholson, S. E., Carter, W., Alexander, W. S., Hilton, D. J., and Nicola, N. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 13261–13265
35. Hwang, M. N., Min, C. H., Kim, H. S., Lee, H., Yoon, K. A., Park, S. Y., Lee, E. S., and Yoon, S. (2007) *Biochem. Biophys. Res. Commun.* **360**, 333–338
36. DiNitto, J. P., Deshmukh, G. D., Zhang, Y., Jacques, S. L., Coli, R., Worrall, J. W., Diehl, W., English, J. M., and Wu, J. C. (2010) *J. Biochem.* **147**, 601–609
37. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Ishiguro, S., Kawano, K., Hanada, M., Kurata, A., Takeda, M., Muhammad Tunio, G., Matsuzawa, Y., Kanakura, Y., and Kitamura, Y. (1998) *Science* **279**, 577–580
38. Isozaki, K., and Hirota, S. (2006) *Curr. Genom.* **7**, 469–475
39. Ning, Z. Q., Li, J., and Arceci, R. J. (2001) *Blood* **97**, 3559–3567
40. Lennartsson, J., Blume-Jensen, P., Hermanson, M., Pontén, E., Carlberg, M., and Rönnstrand, L. (1999) *Oncogene* **18**, 5546–5553
41. Munugalavadla, V., Sims, E. C., Borneo, J., Chan, R. J., and Kapur, R. (2007) *Blood* **110**, 1612–1620