Elongin B/C Recruitment Regulates Substrate Binding by CIS*

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SOCS proteins play a major role in the regulation of cytokine signaling. They are recruited to activated receptors and can suppress signaling by different mechanisms including targeting of the receptor complex for proteasomal degradation. The activity of SOCS proteins is regulated at different levels including transcriptional control and posttranslational modification. We describe here a novel regulatory mechanism for CIS, one of the members of this protein family. A CIS mutant deficient in recruitment of the Elongin B/C complex completely failed to suppress STAT5 activation. This deficiency was not caused by altered turnover of CIS but by loss of cytokine receptor interaction. Intriguingly, no such effect was seen for binding to MyD88. The interaction between CIS and the Elongin B/C complex, which depends on the levels of uncomplexed Elongin B/C, was easily disrupted. This regulatory mechanism may be unique for CIS, as similar mutations in SOCS1, -2, -3, -6, and -7 had no functional impact. Our findings indicate that the SOCS box not only plays a role in the formation of E3 ligase complexes but, at least for CIS, can also regulate the binding modus of SOCS box-containing proteins.

Cytokines regulate multiple biological processes by activating specific cell surface receptor complexes. This leads to a series of signaling events, including activation of the Janus kinase (JAK)2/signal transducer and activator of transcription (STAT), phosphoinositol 3-kinase, phospholipase Cγ, and mitogen-activated protein kinase pathways. The magnitude and duration of a cellular response is determined by the integration of different positive and negative signals. Mechanisms of signal attenuation are diverse and involve different protein families including phosphatases such as PTP-1B (protein tyrosine phosphatase 1B) and TCPTP (T-cell protein-tyrosine phosphatase) (1–5), members of the PIAS (protein inhibitors of activated STATs) (6–8) and SOCS (suppressor of cytokine signaling) families (9–11).

SOCS proteins are induced by a broad range of extracellular ligands and function in a negative feedback loop to modulate signal transduction by multiple cytokine and growth factor receptors (12–14). The eight members of the SOCS family, SOCS1–7 and cytokine-inducible SH2-containing protein (CIS), share a common structure with a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxyl-terminal 40-amino acid module that is known as the SOCS box (9, 15, 16). The SH2 domain is the main determinant of target recognition by the SOCS proteins as it mediates interaction with phosphorylated tyrosine residues on their specific substrates (16–18). In this way, SOCS proteins can suppress signaling by direct competition with signaling molecules for the phosphorylated recruitment sites. SOCS1 and SOCS3 can also inhibit JAK tyrosine kinase activity through their kinase inhibitory region, which is proposed to function as a pseudosubstrate blocking the catalytic cleft of the JAK kinase (19). Finally, SOCS proteins can suppress signaling through proteolytic degradation of the activated receptor complexes. Conserved motives in their SOCS box couple to Elongin B and C (B/C) and Cullin and Rbx proteins, leading to the formation of an E3 ubiquitin ligase complex (20–23) and subsequent ubiquitin marking of the target protein for proteasomal degradation. The functional significance of the association of Elongin B/C with the SOCS box is complex, however, as the SOCS box may also target SOCS proteins themselves for proteasomal degradation (21, 24, 25). Conversely, Elongin B/C binding was also found to stabilize SOCS protein expression (20, 26, 27). Therefore, it is assumed that Elongin B/C binding has a double-edged effect on SOCS proteins: a degrading role by the link with the E3 ubiquitin ligase complex but also a protective function by prevention of proteasomal turnover of the SOCS molecules themselves. There is also evidence that the SOCS box is involved in a SOCS cross-modulatory mechanism, as some SOCS members like SOCS2 can act as negative regulators of other SOCS proteins by targeting them for proteasomal turnover (28–30). Furthermore, we reported previously that the SOCS box of CIS is required for functional interaction with cytokine receptor motifs with a critical role for the single tyrosine residue at position 253 (31).

The founding member of the family, CIS, can inhibit signaling by several cytokine receptors including the erythropoietin receptor (EpoR) and the growth hormone receptor (GHR). CIS suppresses Epo-induced cell proliferation and induces apoptosis of erythroid progenitor cells (32, 33). CIS transgenic mice exhibit growth retardation, suggesting a defect in GH signal transduction (34). Other abnormalities of CIS-overexpressing mice were detected in prolactin and IL-2 signaling pathways,
Overview of the constructs used in the present study

| Name of construct | Template | Cloning vector | Cloning sites | Primers |
|-------------------|----------|----------------|--------------|---------|
| MAPPIT bait constructs | pCEL-Elongin C | pMG2-mElongin C | pCEL | BamHI-NotI |
| pCEL-CIS | pMG2-mCIS | pCEL | SacI-NotI |
| pCEL-CISdB/C | pCEL-mCIS | Mutagenesis | | |
| pCEL-CISdCul | pCEL-mCIS | Mutagenesis | | |
| pCEL-CISdCul | pCEL-mCIS | Mutagenesis | | |
| pCEL-CISdCul | pCEL-mCIS | Mutagenesis | | |
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**Elongin B/C Regulates CIS Function**

Corps. For transfection experiments, HEK293-T cells were freshly seeded in 6-wells plates and transfected overnight with ~2.5 μg of plasmid DNA using a standard calcium phosphate precipitation procedure. The pMET7-SVT construct was used to normalize for the amount of transfected DNA and loading of the transcriptional and translational machinery. The next day, cells were washed with phosphate-buffered saline, transferred to a 96-well plate, and left untreated or stimulated with ligand for at least 24 h. Recombinant mouse leptin and human erythropoietin were purchased from R&D Systems. Luciferase activity from triplicate samples was measured by chemiluminescence in a TopCount luminometer (Canberra-Packard) and expressed as -fold induction (stimulated/nonstimulated). Ba/F3 cells were grown in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (Cambrex Corp.) and 1 ng/ml mIL-3 (Biogen). Transfection of the cell line was done by electroporation (300 V, 1500 microfarads). 48 h after transfection, cells were simultaneously starved (serum and mIL-3 removed) and the cells were stimulated with 1 ng/ml mIL-3 overnight. Activation of the pGL2-SPI2.1-luciferase reporter was measured with the Topcount luminometer (Canberra-Packard).

**Western Blot Analysis and Co-immunoprecipitation**—Transfected HEK-293-T cells were lysed in modified radioimmune precipitation assay buffer (200 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.05% SDS, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM Na3VO4, 1 mM NaF, 20 mM β-glycerophosphate and Complete™ protease inhibitor mixture (Roche Applied Science)). Lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C and incubated with a mixture (10 μg/mL EpoR and biotin-QRQPSVKY(P)ATLVSNDK for Tyr985 in the leptin receptor (LR). Synthesis and purification of the biotinylated (phospho)tyrosine peptides and coupling to streptavidin-agarose beads was described previously (48).

**Pulse-Chase Analysis**—HEK-293-T cells were transfected with 1.0 μg of plasmid DNA using a standard calcium phosphate precipitation procedure. At 40 h after transfection, cells were washed in phosphate-buffered saline and incubated for 1 h in Met/Cys-free Dulbecco’s modified Eagle’s medium (Invitrogen). Cells were then pulsed with Dulbecco’s modified Eagle’s medium containing 0.15 mCi/ml [35S]Met/Cys (PerkinElmer Life Sciences) for 30 min and chased by incubation in unlabeled medium for various times. Cells were lysed in buffer (1 mM EDTA, 150 mM NaCl, 50 mM Tris, 1% Triton X-100, pH 7.5), and the amount of 35S-labeled protein was determined by immunoprecipitation using anti-FLAG tag antibodies coupled to Sepharose (M2 beads, Sigma) followed by gel electrophoresis and autoradiography. Detection and quantification was carried out using a phosphorimager (Typhoon) and associated software.

**Gel Filtration Chromatography**—Approximately 106 Ba/F3 cells were stimulated with 10 ng/ml IL-3 for 1 h at 37 °C and lysed in 3-mI lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8.2, 8 mM EDTA, 0.875% Brij 97 (Sigma-Aldrich), 0.125% Nonidet P-40, 1 mM Pefabloc, and protease inhibitor mixture set III (Calbiochem). Lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C, quantified using the Bradford method, and applied to a calibrated Superdex 75 FPLC gel filtration column (GE Healthcare) run by an advanced protein purification system apparatus (APPS; Waters). The running buffer consisted of 50 mM Tris-HCl pH 7.4, and 150 mM NaCl. After trichloroacetic acid precipitation of equal volumes of collected fractions, the samples were subjected to SDS-PAGE and immunoblotting using the indicated antibodies.

**Modeling Method**—Homology models for CIS, based on the crystal structures of SOCS2 (49) or SOCS4 (50), were created as described previously (39).
RESULTS

CIS Activity Depends on Elongin B/C Binding—To investigate the role of Elongin B/C and Cullin5 binding on CIS function, we generated CIS variants lacking their respective recruitment sites: CISdB/C (L222Q,C226Q) (20) and CISdCul (L240PLP244-AAAA) (51) (Fig. 1A). The CISdB/C mutant was designed to preserve as much as possible the structure of the protein. Leu222 and Cys226 residues, which were part of a helix, and contact Elongin C were replaced with uncharged hydrophilic glutamine residues known to have a high helix propensity. Similar results were obtained with a CISdB/C mutant in which Leu222 and Cys226 were substituted by alanine (data not shown). CIS activity was evaluated by a STAT5-dependent reporter assay in HEK293-T cells transiently transfected with the human EpoR expression vector and CIS, SOCS2, or mutant constructs. Although wild type CIS clearly suppressed reporter activity as expected, the CISdB/C derivative was almost completely unable to impair reporter induction (Fig. 1B). In contrast, the CISdCul variant was still fully functional, indicating that the failure of receptor inhibition observed for CISdB/C could be attributed specifically to the loss of Elongin B/C recruitment. Only binding of the Elongin B/C complex, and not the complete E3 complex, is thus a critical requirement for CIS function. As a control, we also included the Y253F loss-of-function CIS mutant (31). In evident contrast, a similar B/C box mutation in the highly related SOCS2 protein (SOCS2dB/C, L163Q,C167Q) showed no effect on STAT5 signaling. To verify the effect of Elongin B/C depletion on CIS activity, we also co-expressed SOCS4, which is not an inhibitor of EpoR signaling. Scavenging of Elongin B/C by SOCS4 antagonized CIS-dependent inhibition in a dose-dependent manner (data not shown).

To examine the function of the CIS mutants in a more physiological setup, STAT5-dependent reporter assays measuring IL-3 signaling were performed in the murine Ba/F3 pro-B cell line. Again, we observed that the inhibitory effect of CIS was completely dependent on an intact B/C box (Fig. 1C).

Elongin B/C Recruitment to CIS Is Required for Receptor Substrate Binding—It is well established that the interaction of SOCS proteins with their receptor targets depends on their SH2 domains (16, 18). In the case of CIS, we recently demonstrated that the carboxyl-terminal Tyr253 residue is also required for interaction with phosphotyrosine motifs in cytokine receptors (31). Because disruption of Elongin B/C binding abrogated CIS function, we next investigated the involvement of Elongin B/C
recruitment in substrate binding. To address this question we used the mammalian protein-protein interaction trap (MAPPIT) method, a strategy designed to analyze protein-protein interactions in mammalian cells. In MAPPIT, a bait protein is carboxyl-terminally linked to a chimeric receptor that is deficient in STAT3 recruitment, and a prey protein is fused to a part of the glycoprotein 130 (gp130) chain containing four functional STAT3 recruitment sites. Co-expression of an interacting bait/prey pair leads to functional complementation of STAT3 activity and induction of a STAT3-responsive luciferase reporter.

FIGURE 2. Elongin B/C binding is critical for interaction of CIS with receptor motifs. A diagrammatic presentation of the MAPPIT configurations used in this study. The lower panel shows the bait and prey chimeras. The right panel shows a variant of the MAPPIT technique wherein the LR itself functions as bait protein. See "Results" for an explanation of the MAPPIT technique. B, HEK293-T cells were transiently cotransfected with bait plasmids encoding the LR (YYF), the EpoR Tyr402 or the GHR Tyr595 motif and prey plasmids encoding RNF41 as positive control, CIS, SOCS2, or derived mutant constructs combined with the STAT3-responsive pX2d2-rPAPI-luciferase reporter. 24 h after transfection, cells were left untreated or were stimulated with leptin (100 ng/ml) or Epo (5 ng/ml) overnight. Luciferase data of triplicate measurements are expressed as fold induction (stimulated/nonstimulated ratio). Expression of the FLAG-tagged prey proteins was evaluated by immunoblotting using anti-FLAG antibody. C, (phospho)peptide affinity chromatography. HEK293-T cells were cotransfected with FLAG-tagged CIS, SOCS2, or mutant derivatives. The lysates were incubated with phosphorylated or nonphosphorylated peptides corresponding to the Tyr402 motif of the EpoR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody. D, HEK293-T cells were transiently cotransfected with bait plasmids encoding the GHR Tyr595 motif or MyD88 and prey plasmids encoding CIS or mutants combined with the STAT3-responsive pX2d2-rPAPI-luciferase reporter. MAPPIT signaling was assayed as described in B. E, verification of the interaction pattern of the CIS mutants. In the upper panel, HEK293-T cells were cotransfected with plasmids encoding the simian virus 40 large T-antigen (SVT) as a negative control, Elongin B, CIS, or derived mutant prey constructs combined with the indicated bait constructs and the pX2d2-rPAPI-luciferase reporter. MAPPIT signaling was assayed as described in B. In the bottom panels, the binding modes of the CIS mutants are demonstrated by co-immunoprecipitation experiments. Lysates of HEK293-T cells transfected with FLAG-tagged CIS or CISdDB/C were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-Elongin C (left panel). HEK293-T cells were cotransfected with combinations of FLAG-tagged Cullin5 and E-tagged CIS, CISdDB/C or CISdCul, and the lysates were immunoprecipitated with anti-E and then immunoblotted with anti-FLAG (right panel).
ase reporter. MAPPIT permits the detection of both modification-independent and phosphorylation-dependent interactions. The MAPPIT configurations used in this manuscript are described in Fig. 2A. Functional expression of the different bait constructs was assessed by measuring interaction with the JAK2-binding RNF41 (ring finger protein 41) prey. The CIS Y253F prey was again used as a loss-of-function control.

As examples of known interaction partners of CIS and SOCS2, we used the intracellular receptor tyrosine motifs Tyr402 of the EpoR (42) and Tyr595 of the GHR (39) as baits. We also analyzed interactions with the LR by mutating the STAT3-recruiting Tyr1138 to Phe (LR (YYF)). This way, MAPPIT analysis of interactions at positions Tyr985 and Tyr1077, known to recruit SOCS proteins (47, 48, 52), is possible using the full-length LR. HEK293-T cells were cotransfected with the aforementioned baits and prey constructs encoding the different CIS and SOCS2 variants, combined with the STAT3-responsive rPAPI luciferase reporter. Fig. 2B shows that elimination of the Elongin B/C binding site in the CIS prey caused complete loss of binding to all studied receptor motifs. In contrast, deletion of the conserved B/C box in the SOCS2 prey did not significantly affect interaction with the baits. The observed interaction patterns with the EpoR Tyr402 bait were confirmed by phosphopeptide affinity chromatography. Complete loss of binding to the phosphorylated Tyr402 of the EpoR was observed for the CISdB/C or Y253F mutants, whereas no effect was seen for a similar SOCS2dB/C mutant (Fig. 2C). In analogy with the observed interaction pattern of the CIS Y253F mutant (31), deletion of the B/C box did not affect interaction with the Toll-like receptor adaptor, MyD88 (Fig. 2D). This implies that the structural integrity of the CIS prey is maintained upon deletion of the Elongin B/C recruitment site. In Fig. 2E we integrated binding controls for the CIS mutants. The top panel shows a MAPPIT experiment demonstrating that the CISdB/C, but not the CISdCul prey, is incapable of interacting with an Elongin B/C.
Elongin B/C Regulates CIS Function

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**FIGURE 3. CIS half-life determination by metabolic labeling and pulse-chase analysis.** HEK293-T cells were transiently transfected with combinations of FLAG-tagged CIS (with/without Elongin B/C) or FLAG-tagged CISdB/C. 40 h after transfection, the cells were washed and pulse-labeled with 35S-labeled methionine and cysteine for 30 s. The cells were then washed three times and chased for the indicated periods of time. Thereafter, the cells were lysed, and the CIS proteins were immunoprecipitated with anti-FLAG antibody followed by SDS-PAGE and autoradiography. The relative intensity of the CIS bands was normalized and quantified by densitometry. The graph shows processed data of the pulse-chase experiment in which the initial maximal expression was defined as 100%.

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Elongin C bait. Similar data were obtained in a reciprocal setting: a complete loss of binding with the Elongin B and C (B/C) preys and Cullin5 prey is seen for the CISdB/C bait, whereas interaction of only the Cullin5 prey is lost for the CISdB/C mutation. Finally, the bottom panels (Fig. 2E) show co-immunoprecipitation experiments demonstrating loss of interaction with the endogenous Elongin C and/or FLAG-tagged Cullin5 upon mutation of the B/C motif or the Cullin box in CIS.

**Loss of Substrate Binding by CISdB/C Is Not Due to an Effect on CIS Stability**—Next we wanted to verify whether an altered protein half-life time due to the CISdB/C mutation could account for the loss of substrate binding. To this end, we performed pulse-chase experiments in which HEK293-T cells expressing FLAG-tagged CIS (with or without Elongin B/C) or FLAG-tagged CISdB/C were metabolically labeled with 35S-labeled methionine and cysteine. The cells were chased for the indicated periods, and after cell lysis the different CIS constructs were immunoprecipitated with FLAG antibody followed by gel electrophoresis and autoradiography. Protein half-life times were estimated following quantification of the band intensities. The graph in Fig. 3 shows processed data of the pulse-chase experiment in which the initial maximal expression was defined as 100%. The estimated half-life was 1 h for CIS and 1 h, 30 s for CISdB/C, respectively, ruling out an effect of protein stability on the interaction assays. Notably, Elongin B/C co-expression also significantly prolonged CIS half-life time, indicating a stabilizing effect of Elongin B/C resulting in elevated CIS levels. Similar results were obtained by performing degradation assays with the translation inhibitor cycloheximide (data not shown).

**CIS Elongin B/C Dependence for Substrate Interaction Is Unique among SOCS Proteins**—We next questioned whether other SOCS family members also display a similar dependence on Elongin B/C binding for substrate recognition. We used MAPPIT to examine the binding of SOCS6 and SOCS7 or of their respective Elongin B/C deletion mutants on the LR (YYF) bait. Similar to SOCS2, the dB/C mutation in SOCS6 or SOCS7 did not alter the interaction pattern (Fig. 4A). Other readouts were used for SOCS1 and SOCS3 as these inhibit the MAPPIT assay because of their JAK suppressive activity. The interaction of SOCS1 with JAK2 was analyzed by co-immunoprecipitation. As shown in Fig. 4B, SOCS1 and its dB/C mutant bind equally well to JAK2. For SOCS3, no effect was seen for the SOCS3dB/C mutant in a STAT5-dependent reporter assay, implying normal interaction with the EpoR (Fig. 4C). Similarly, normal binding was seen for the SOCS3dB/C mutant in a phosphopeptide affinity chromatography experiment using the phosphorylated Tyr<sup>595</sup> motif of the LR (Fig. 4D).

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CIS and SOCS2 Display Different Binding Properties for the Elongin B/C Complex—Co-immunoprecipitation experiments demonstrated that CIS coprecipitates less endogenous Elongin C than SOCS2 (Fig. 5A). We next compared the relative binding properties of CIS and SOCS2 for Elongin C in a MAPPIT setup. To this end, the interaction of a SOCS2 or CIS prey with the Elongin C bait was assessed by cotransfection of increasing amounts of wild type CIS or SOCS2, respectively (Fig. 5B). In contrast to SOCS2, which strongly competed with the CIS prey for binding to Elongin C, CIS could not interfere with the interaction between the SOCS2 prey and the Elongin C bait, suggesting a lower binding affinity of CIS for Elongin C. Thus, the CIS interaction with the Elongin B/C complex is easily disrupted, and accordingly CIS function may depend on the availability of a free Elongin B/C pool within the cell.

**Elongin B/C Levels Can Determine CIS Activity**—To verify the concept that CIS function is regulated by the intracellular level of free Elongin B/C complex, we performed several experiments. First, we used MAPPIT to examine whether co-expression of Elongin B/C in HEK293-T cells could enhance CIS substrate binding. As shown in Fig. 6A, this is clearly the case when using the GHR Tyr<sup>595</sup> motif as bait, whereas Elongin B/C co-expression did not induce a stronger binding of the SOCS2 prey to the GHR receptor motive. This implicates the existence of a pool of free CIS prey that becomes activated upon Elongin B/C recruitment. In addition, phosphopeptide affinity chromatography demonstrated that co-expression of Elongin B/C increased CIS interaction to a phosphorylated Tyr<sup>402</sup> motif of the EpoR, whereas no enhanced SOCS2 binding was observed. The binding levels were determined by quantification of the band intensities, which were normalized with the expression levels, compensating for the enhancing effect of cotransfected Elongin B/C on protein stability (Fig. 6B).

We next examined whether an unbound endogenous CIS fraction exists. This was evaluated in the physiological background of BaF/3 cells. Lysates of IL-3-stimulated Ba/F3 cells...
were separated by gel filtration chromatography over a Superdex 75 PG16/60 column and the fractions containing CIS and Elongin C were identified by immunoblotting. As shown in Fig. 6C the majority of CIS eluted in fractions 32–34, corresponding to monomeric CIS (44 kDa), and only a subset of the total CIS pool co-eluted with Elongin C (>44 kDa). The different bands revealed by CIS immunodetection likely correspond to different forms of CIS, as no bands could be detected in lysates of starved Ba/F3 cells (data not shown). Two bands may correspond to the mono- (37 kDa) and non-ubiquitinated (32 kDa) forms of CIS.

FIGURE 4. Substrate interaction of other SOCS proteins is not Elongin B/C-dependent. A, MAPPIT analysis of the binding modus of SOCS6 and SOCS7 (dB/C) preys with the leptin receptor. HEK293-T cells were transiently cotransfected with plasmids encoding the indicated prey constructs combined with the LR(YYF) bait and the pXP2d2-rPAPI-luciferase reporter. MAPPIT signaling was assayed as described in the legend Fig. 2B, co-immunoprecipitation analysis of the interaction between JAK2 and SOCS1(dB/C). HEK293-T cells were transiently transfected with combinations of JAK2 and E-tagged SOCS1, SOCS1dB/C, or MyD88 as negative control. Cell lysates were immunoprecipitated (IP) with anti-E tag and subsequently immunoblotted (IB) with anti-JAK2. C, effect of the mutation of the B/C box on SOCS3 inhibition of EpoR signaling. HEK293-T cells were transfected with expression vectors for SOCS3, CIS, or their dB/C deletion mutants combined with the human EpoR and the β-casein-derived luciferase reporter gene. EpoR signaling was assayed as described in the legend for Fig. 1B. D, (phospho)peptide affinity chromatography analyzing the interaction between SOCS3 or its dB/C mutant and the Tyr985 motif of the LR. HEK293-T cells were transfected with FLAG-tagged SOCS3 or SOCS3dB/C, and lysates were incubated with phosphorylated or nonphosphorylated peptides corresponding to the Tyr985 motif of the LR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody.
forms of CIS (35, 53). This provides evidence for the occurrence of a cellular population of free CIS, which can thus be bound and regulated by Elongin B/C.

**DISCUSSION**

SOCS proteins are known to act as the substrate recognition part of a RING-type E3 ubiquitin ligase complex. Association of the SOCS box with the adaptor proteins Elongin B and C mediates further assembly with Cullin and Rbx proteins, resulting in the formation of a multiprotein E3 ligase. This complex will function as a scaffold that presents bound substrate to an E2 ubiquitin-conjugating enzyme, ultimately leading to ubiquitination and degradation of the target molecule. Within the SOCS box, conserved Elongin B/C and Cullin boxes, respectively, mediate Elongin C (20, 54–57) and Cullin2 or -5 recruitment depending on the SOCS family member (51). We report here that deletion of the B/C box completely abrogated CIS function through loss of substrate binding. Deletion of the Cullin5 box had no impact on the inhibitory effects of CIS, indicating that binding of Elongin B/C only, and not the association of a larger E3 complex, is essential for interaction of CIS with its cognate cytokine receptor motifs.

Two mechanisms for CIS inhibition have been proposed: 1) partial inhibition by direct competition with STAT5 for common phosphotyrosine binding sites on the receptor and 2) proteasome-mediated degradation of the receptor-JAK2 signaling complex. In the case of the GHR, this latter mechanism may be coupled to internalization of the activated receptor complex, a critical step preceding termination of receptor signaling (41). SOCS-mediated inhibition of the GHR by competition for shared binding sites with STAT5 was recently ruled out based on the nonoverlapping bindings pattern of CIS and SOCS2 with STAT5 (39). Furthermore, the SOCS box of CIS was found to be essential for the apoptotic effect of CIS on erythroid progenitor cells (33). Our observation that Elongin B/C recruitment cannot be uncoupled from CIS function lends further support for a primary role for the formation of an E3 ligase complex in CIS-mediated signal suppression. Nevertheless, we found that deletion of the Cullin box did not completely abolish the inhibitory functions of CIS in EpoR (and in IL-3R, data not shown) signaling; this is currently being investigated in greater detail.

Much in contrast, no evidence was obtained for a role in substrate binding by the Elongin B/C box of SOCS1, -2, -3, -6, and -7. Because none of the other examined SOCS members was found to depend on Elongin recruitment for interaction with target motifs, this may be a unique feature of CIS. This specific effect seems to parallel the effects observed for the Y253F mutation in the carboxyl-terminal portion of the CIS SOCS box. We previously demonstrated that this mutation also completely abrogated functional interaction with most cytokine receptor-based interaction motifs (31, 39). Again, no such role for conserved tyrosines in the SOCS box was observed for the highly related SOCS2 proteins or for SOCS1 and -3. Mutation of either the Elongin B/C motif or Tyr253 in CIS led to loss of binding to all substrates tested except MyD88. Together, these data suggest that the Elongin B/C mutation and Tyr253 mutation affect receptor binding through a common mechanism.

Two different homology models were built for CIS with a SOCS box orientation as in SOCS2 (Fig. 7A, homology model 1) or SOCS4 (Fig. 7B, homology model 2). Both models exclude a direct interaction of the SOCS box or Tyr253 with the phosphopeptide substrate. Tyr253 shows no direct interaction with the Elongins, and mutation of Tyr253 does not impair Elongin B/C binding (31). In both models, Tyr253 is found in the interface between the SOCS box and the SH2 domain, suggesting that it might mediate an allosteric regulation of substrate binding by the SOCS box domain. We hypothesize that the correct position of Tyr253 is critical for substrate binding and that this correct position is induced by structural changes in the SOCS box upon binding of Elongins. Similar structural rearrangements take place in yeast Elongin C upon binding of a SOCS box.
peptide from the von Hippel-Lindau (VHL) protein (58). VHL requires Elongin B/C binding to adopt a stable structure (59, 60) and stable expression. The deletion of the Elongin binding site in CIS appeared to have no drastic effects on the structural integrity of the SH2 domain protein, because B/C box-independent interactions of CIS were still observed with MyD88. We verified whether the loss-of-function of the CISdB/C mutant was not due to an altered half-life time. The particular CIS mutant appeared to be even more stable than the wild type protein. However, Elongin B/C co-expression significantly extended the half-life time of wild type CIS, indicating that a stabilizing effect of Elongin B/C on CIS does exist.

**FIGURE 6. Elongin B/C levels can determine CIS activity.** A, the binding potency of the CIS prey depends on Elongin B/C levels in MAPPIT. HEK293-T cells were transiently cotransfected with expression vectors encoding the pXP2d2-rPAPI-luciferase reporter, the GHR Tyr595 bait, and CIS or SOCS2 (dB/C) preys with or without Elongin B/C co-expression. MAPPIT signaling was assayed as described in the legend for Fig. 2B. B, (phospho)peptide affinity chromatography. HEK293-T cells were transfected with FLAG-tagged CIS or SOCS2 with or without Elongin B/C co-expression. Lysates were incubated with immobilized phosphorylated or nonphosphorylated peptides corresponding to the Tyr402 motif of the EpoR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody. CIS and SOCS2 binding was quantified using the Odyssey infrared imaging system (Li-Cor); these values were normalized for the respective expression levels. The data shown (upper panel) are representative of three independent experiments. The lower graph shows processed data representing the average value and standard deviations of three experiments. The initial binding level was defined as 1. C, evidence for uncomplexed CIS in Ba/F3 cells. Lysates of IL-3-activated Ba/F3 cells (10^8 cells) harboring endogenous CIS were size-fractionated by gel filtration on a Superdex 75 PG16/60 column. The individual fractions were subjected to SDS-PAGE and immunoblotting using anti-CIS and anti-Elongin C antibody. As a standard, a mix of proteins of defined molecular weights was also fractionated on the same column, allowing an estimation of the molecular weight of the different fractions.
In co-immunoprecipitation experiments CIS appeared to display a lower affinity for Elongin C than SOCS2. Furthermore, MAPPIT competition experiments showed that SOCS2 overexpression easily interfered with the interaction between CIS and Elongin C, a phenomenon that appeared to be unidirectional. This sensitivity of CIS for Elongin recruitment suggests an underlying regulatory mechanism. Conceivably, CIS activity may be down-modulated by SOCS2 in a dual way. First, SOCS2 is induced at a later stage of post-receptor activation and may scavenge Elongin B/C from CIS, leading to loss of substrate binding. Second, SOCS2 can interact with unbound CIS leading to degradation of the free CIS pool. We reported previously that this involves the SOCS box of CIS as the interaction domain for SOCS2 and also the SOCS box of SOCS2 as the template for building the E3 ligase complex (29).

Lending further support for regulation at this level is our observation that CIS activity depends on the levels of Elongin B/C within the cell. Using MAPPIT we demonstrated that co-expression of Elongin B/C increased CIS prey interaction with a receptor motif, whereas no such effect was seen for SOCS2 prey binding. We also provided evidence for the existence of monomeric CIS protein in BaF/3 cells by gel filtration analysis. These findings suggest that the activity of free CIS protein can be modulated functionally by Elongin B/C and that the availability of unbound Elongin B/C complex will determine CIS activity.

Thus far, little is known about the mechanisms that determine free Elongin B/C levels in the cell, and more detailed studies are clearly required. Elongin B/C may be part of different multiprotein complexes including the RNA polymerase II (61, 62) machinery and a large subfamily of E3 ubiquitin ligases. This encompasses the VHL tumor suppressor complex (63) and more than 70 proteins harboring a SOCS box in the human genome (according to the Pfam database). As each of these could possibly sequester the Elongin B/C complex, it is clear that depending on the induction pattern, subcellular localization, and relative binding affinities of all of these SOCS box-containing proteins, a competition for Elongin B/C will occur. Of note, viral genomes also can encode proteins that recruit Elongin B/C, e.g. human immunodeficiency virus-1 Vif (viral infectivity factor), which suppresses the antiviral activity of APOBEC3G (64). We also note that inappropriately elevated SOCS (65–70) or Elongin C (71) levels can be found in several oncologic disorders.

We can only speculate on the physiological reason behind this built-in molecular on/off switch in CIS. It is intriguing that different effects are observed for different substrates, CIS binding of several cytokine receptors is under tight control, whereas no effect is seen for the interaction with MyD88. Perhaps, this observed Elongin B/C dependence may function as a “safety lock” to ensure complete suppression of signaling, as only the CIS molecules that recruit an E3 ligase complex will be able to participate in the inhibition. Conceivably, such tight control may be required for the vital processes that are modulated by CIS, such as GHR-mediated somatic growth and cellular metabolism. The remarkable discrepancy observed between the lack of phenotype of CIS knock-out mice and the defective phenotype in growth, mammary gland development, and immune effects of CIS transgenic mice (34, 72) may also suggest the high risk of unrestrained CIS activity.

In conclusion, our findings further underscore the functional complexity of the SOCS box domain. In addition to its role in protein turnover, the SOCS box domain appears to be involved also in the regulation of substrate binding.

Acknowledgments—We gratefully acknowledge the technical support received from Marc Goethals for peptide synthesis and from Hans Caster in assisting with the gel filtration experiments.
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