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The SOCS box encodes a hierarchy of affinities for Cullin5: implications for ubiquitin ligase formation and cytokine signalling suppression

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Summary

The SOCS (Suppressors of Cytokine Signalling) family of proteins inhibit the cytokine induced signalling cascade, in part by promoting the ubiquitination of signalling intermediates that are then targeted for proteasomal degradation. This activity relies upon an interaction between the SOCS box domain, the adapter complex elonginBC and a member of the Cullin family, the scaffold protein of an E3 ubiquitin ligase. Here we dissect this interaction, *in vitro*, using purified components. We show that all eight SOCS proteins bound Cullin5, but required prior recruitment of elonginBC. Neither SOCS nor elonginBC bound Cullin5 when in isolation. Interestingly, the affinity of each SOCS/elonginBC complex for Cullin5 varied by two orders of magnitude across the SOCS family. Unexpectedly, the most potent suppressors of signalling, SOCS-1 and SOCS-3, bound most weakly to the E3 ligase scaffold, with affinities 100- and 10-fold lower, respectively, than than the rest of the family. The remaining six SOCS proteins all bound Cullin5 with high affinity ($K_D \sim 10$ nM), due to a slower off-rate, and hence a longer half-life of the complex. This difference in affinity may reflect a difference in mode-of-action, as only SOCS-1 and -3 have been shown to suppress signalling using both SOCS box dependent and independent mechanisms. This is not the case with the other six SOCS proteins and our data imply the existence of two distinct sub-classes of SOCS proteins, with a high affinity for Cullin5, the E3 ligase scaffold, possibly reflecting complete dependence upon ubiquitination for suppression of cytokine signalling.

Keywords

SOCS; Cytokine Signalling; Ubiquitin ligase; Elongin; Cullin

Introduction

The Suppressors of Cytokine Signalling (SOCS) proteins were first isolated on the basis of their ability to inhibit the intracellular signal transduction pathways initiated by cytokine stimulation. Cytokines act through binding the appropriate membrane-bound receptor and inducing receptor dimerisation or re-orientation. This allows Janus Kinases (JAKs), associated

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with the intracellular domains of the receptor, to initiate a cascade of tyrosine phosphorylation events which eventually results in STAT (Signal Transducers and Activators of Transcription)-mediated upregulation of cytokine responsive genes[1]. The SOCS proteins themselves are transcriptional targets of STATs and they then act to down-regulate the intracellular signalling cascade as part of a negative feedback loop[2-4]. In humans, the SOCS family comprises eight members: SOCS1-7 and CIS (Cytokine Inducible SH2 domain containing protein)[5]. Many of these are induced by a wide range of cytokines and are potent regulators of intracellular signalling[6]. The eight SOCS proteins have in common a central SH2 domain and a short C-terminal domain called the SOCS box as well as an N-terminal domain of varying length and often unknown function. The SH2 domains and N-terminal regions, at least in some cases, down-regulate signalling via direct or competitive binding inhibition of signalling intermediates[7-12]. In contrast, the SOCS box acts via a different mechanism: it interacts with cellular ubiquitination machinery[13-18] to promote the degradation of bound signalling pathway intermediates.

The hypothesis that SOCS proteins might induce substrate ubiquitination arose from the similarity of the SOCS box to the α-domain of the von-Hippel Lindau (VHL) protein. The VHL α-domain binds elonginBC and associates with an E3 ubiquitin ligase to induce Hypoxia Inducible Factor-1α (HIF-1α) degradation[19]. E3 ubiquitin ligases, in the presence of ubiquitin-activating and conjugating enzymes (E1 and E2 respectively) catalyse the covalent addition of ubiquitin to lysine sidechains on target proteins[20]. Polyubiquitination of proteins then leads to their recognition and degradation by the proteasome. According to this model, the SOCS proteins, like VHL, act as substrate adapters. They bind to the E3 ligase through the SOCS box and thereby induce the ubiquitination of signalling intermediates bound to their SH2 and N-terminal domains. As all eight SOCS proteins contain a central SH2 domain, any tyrosine phosphorylated signalling intermediate, such as phospho-JAK, phospho-STAT and even phosphorylated receptors, are potential substrates.

In support of this hypothesis, SOCS3 was shown to interact with Cullin5 and Rbx2[21]. Cullins are a family of scaffold proteins for the Cullin-Ring type E3 ligases (CRLs). The C-terminal domain of Cullin5 associates with Rbx2, a ring finger protein which acts as a docking site for the E2 enzyme, whilst the N-terminal domain binds to a SOCS protein, via the adapter complex, elonginBC. This would then allow the ubiquitination of tyrosine phosphorylated signalling molecules bound to SOCS. A wider family of SOCS box containing proteins is encoded in eukaryotic genomes with different upstream protein-protein interaction domains, such as WD40 domains, ankyrin repeats, GTPase domains and SPRY domains[11]. In an elegant study, Kamura *et al*, showed that several of these also bound Cullin5/Rbx2 making this the assumed target for all SOCS box-containing proteins[21]. However, there is still some uncertainty regarding SOCS1, as no interaction with Cullin5, or Cullin2 was detected using endogenous protein[21] although a SOCS1-Cullin2 interaction has been implied using overexpression systems[18]. In addition SOCS1 has been shown to catalyse the degradation of the TEL-JAK oncoprotein, vav, IRS-1 and IRS-2[18]. The specificity for Cullin5/Rbx2 implies that the SOCS box is functionally distinct from the highly similar VHL box domain which targets proteins to Cullin2/Rbx1.

In contrast to SOCS1, ubiquitin ligase activity has not been demonstrated for SOCS2-7 and CIS, therefore we aimed to address the question as to whether all SOCS proteins are able to form active E3 ligases. Based on the current model the first step in E3 ligase formation would be an interaction with Cullin5. In this report we show that the SOCS boxes of all eight SOCS proteins, including SOCS1, can interact with Cullin5. This interaction is a two-step process that first requires elonginBC association. Our biophysical analyses show that the SOCS proteins can be divided into two distinct classes based on their affinity for Cullin5. SOCS1 and SOCS3 bind relatively weakly to Cullin5 whilst the rest of the family associates more tightly

with a dissociation constant of ~ 10^{-8} M and a correspondingly longer half-life (100-200 s). Quantitative affinity measurements on SOCS box mutants support the findings of Kamura *et al.*,[21] by showing that the high affinity for Cullin5 is encoded by four residues L-P-[I/L]-P, the Cul box. Interestingly, although a number of SOCS proteins are presumed to act partially via proteasome-independent mechanisms, this has only been shown directly for SOCS1 and SOCS3. Our finding that the other six SOCS proteins form a subclass of the SOCS family that binds with much higher affinity to the ubiquitin ligase scaffold, is consistent with an hypothesis in which this subclass relies solely on ubiquitin/proteasome dependent processes in order to suppress signalling.

Results

The SOCS box domains of all members of the SOCS family bind elonginBC

To investigate whether all eight SOCS proteins utilize a Cullin5 based E3 ligase, we undertook *in vitro* binding studies using purified recombinant proteins. This approach has the advantage over co-precipitation studies of allowing affinity measurements as well as showing that an interaction is direct, rather than being mediated by another molecule. Previous attempts at such analyses have been hampered by the difficulty of producing correctly folded and soluble SOCS proteins. As we showed recently, the SOCS box of SOCS3 acts as an independent domain that interacts with Cullin5 with identical affinity and enthalpy to the full-length protein[22] and we hypothesised that the SOCS box domains from other SOCS proteins would behave similarly. As elonginBC is known to moderate the SOCS-Cullin association the SOCS box domains from SOCS1-7 and CIS were cloned, co-expressed with elonginB and elonginC in *E. coli* and purified. GST-pulldown data in Figure 1a show that the SOCS box from every member of the SOCS family formed a stable complex with elonginBC. Once the GST was proteolytically removed, these complexes could be further purified by gel-filtration chromatography (Fig. 1b, Fig. S1). As described elsewhere[22], the affinity of SOCS proteins for elonginBC cannot be assessed using standard titration techniques as elonginBC produced in isolation, is in a state that will not interact with SOCS. Nevertheless, the SOCS box/elonginBC ternary complexes were all stable when assessed by gel filtration chromatography, even at concentrations as low as ~10 μM (data not shown), suggesting that the interaction was of reasonable affinity. The interactions of SOCS2, SOCS3 and SOCS4 with elonginBC have all been characterised structurally[23,24]. Mutational analysis of the SOCS3 box showed that the first 12 residues of the SOCS box were necessary and sufficient for the elonginBC interaction and that a conserved leucine, Leu4, was absolutely required for binding. NMR analysis showed that this leucine has a distinctive chemical shift, only when bound to elonginBC, due to its proximity to Tyr76 of elonginC which induces a ring-current-induced downfield shift. Thus the chemical shift of Leu4 is a useful marker of binding. NMR analysis of all eight SOCS box domains bound to elonginBC showed a similar phenomenon (Figure S1). In each case there was an amide resonance at 11-12 ppm suggesting that Leu4 of each SOCS box is involved in binding and located in a very similar environment in the bound state.

All ternary SOCS box/elonginBC complexes bind Cullin5

Having established that all SOCS box domains bound to elonginBC we next investigated whether the ternary complexes bind Cullin5. As the substrate-binding site on cullin proteins is located within the N-terminal domain which can fold properly in the absence of the C-terminal domain[22,26], a 45-kDa N-terminal fragment of cullin5 was used in these studies. This domain was expressed in *E. coli* as a recombinant GST fusion protein. As shown in Figure 2, all eight ternary SOCS box/elonginBC complexes bound cullin5. These SOCS box domain/elonginBC/cullin5 quaternary complexes all co-eluted when analysed by gel filtration (data not shown). Interestingly, elonginBC alone did not bind Cullin5, nor did a SOCS3 box$_{\Delta C28}$/elonginBC complex which lacks the predicted cullin binding motif (LPXP). In addition, in the

absence of elonginBC no direct interaction of any SOCS box domain with cullin5 was observed (data not shown). Together, these data show that the SOCS box from all SOCS proteins can bind cullin5 in a two-step process that first requires association with elonginBC.

The affinity for Cullin5 describes two distinct subclasses of SOCS proteins

Although all eight SOCS proteins could interact with Cullin5 as measured using GST pull-down methodology, this gives no information on the affinity of each interaction. Affinity is a powerful determinant of biological activity and intracellular signalling pathways and networks are fine-tuned through the affinity of each protein-protein interaction they are made up of [25]. Given the relatively few examples of SOCS based E3 ligase activity shown to date, and given that Cullin binding is a necessary step in E3 ligase formation, we used Isothermal Titration Calorimetry (ITC) to determine the affinity with which each SOCS box domain, in complex with elonginBC, could bind Cullin5. In these experiments 100 μM GST-tagged Cullin5 (N-terminal domain) was titrated, in 10 μl aliquots, into pre-formed SOCS box/elonginBC ternary complexes (10 μM). The heat of dilution of GST-Cullin5 was assessed by titration into buffer alone and this was subtracted from the final result. Each titration was performed in duplicate and the values of these were used to calculate the average and range, rather than deviations in curve fitting of one experiment, which are often artificially low. Several experiments were also repeated using Cullin5 that had the GST removed by thrombin cleavage in order to ensure that the presence of GST did not lead to dimerisation induced artefacts (Figure S2). As shown in Figure 3, SOCS2, 4, 5, 6, 7 and CIS all bound Cullin5 with high affinity ~ 10^{-8} M. In contrast, SOCS1 and SOCS3 were distinctive, in that they bound 100- and 10-fold more weakly, respectively. SOCS1 binds with only micromolar affinity explaining why Kamura and colleagues could not detect the endogenous interaction[21]. All eight interactions had a low enthalpy when performed at 298K however this was due to large heat capacity (ΔCp) associated with the interaction as each interaction investigated at 278K was endothermic in nature. A large ΔCp is often characteristic of binding driven by hydrophobic interactions.

Although SOCS-4, -5, and -7 contain large C-terminal extensions in their SOCS box domains, placing them in a different structural class[23,24] this was not reflected in their affinity for Cullin5, as they bound with near identical affinity to SOCS-2, -6 and CIS which do not contain a C-terminal extension. Our data suggest two functionally based subgroups of SOCS proteins based on their affinities for Cullin5 which separates SOCS1 and SOCS3 from the rest of the family.

The low affinity of SOCS1, but not SOCS3, for Cullin5 is due to a non-canonical Cul box sequence

The Cullin binding motif, previously defined by Kamura *et al.*,[21] is located in the C-terminal half of the SOCS box, particularly an L-P-X-P motif located in this region. The vast majority of SOCS proteins, and the wider family of SOCS box-containing proteins have L-P-I/L-P as this motif. SOCS1 and SOCS3 are distinct in containing I-P-L-N and L-P-G-P respectively (Figure 4a). We therefore constructed mutants of these two SOCS boxes, in which both sequences were replaced by L-P-L-P, the canonical sequence. These mutant SOCS boxes were co-expressed with elonginBC and their affinities for Cullin5 examined by ITC. Both mutants bound Cullin5 with significantly lower enthalpy, making analysis at 298K difficult (Figure S3). The analyses were therefore repeated at 27 8K where the reaction has a greater absolute enthalpy, even though it is endothermic in nature. As shown in Figure 4b, mutating the Cul5 box to L-P-L-P completely restored SOCS1 affinity for Cullin5 to 10 nM but had no effect on SOCS3 affinity. We recently showed that the SOCS3 box is not completely structured in the absence of Cullin5, even when bound to elonginBC[22]. Therefore there may be an entropic cost to SOCS3/elonginBC binding to Cullin5 compared to other SOCS proteins which could explain its lower affinity. Whilst mutating IPLN to LPLP in the SOCS1 box restored cullin5 binding affinity, the mutant bound with significantly lower enthalpy and consequently, a more

favourable entropy. This is consistent with the direct contact that the terminal proline is predicted to make to cullin5 but may also be explained by the extra rigidity, and hence lower entropic cost to binding, provided by a proline residue. Analysis of the available SOCS/elonginBC structures[22-24] and a comparison with the SCF E3 ligase structure[26] suggests that elonginC and the SOCS box bind to each other in a way that forms a continuous surface between elonginC and the C-terminal proline of the LPXP motif of the SOCS box (Fig. 4c). It is this surface, rather than elonginC or the SOCS box alone that is the Cullin5 interacting unit. Although the majority of this binding surface arises from elonginC a direct elonginBC-Cullin interaction was too weak to detect (> 10 μM) in the absence of the SOCS box. Therefore it is clear that the SOCS box itself contributes significantly to the interaction, being (a) required for association and (b) encoding a 100-fold difference in affinity across the family.

The variation in Cullin5 affinity arises from differences in the off-rate of the complex

The high affinity of the majority of the SOCS/elonginBC complexes for Cullin5 implies either a faster on-rate (association rate) or else a slower off-rate (dissociation rate) for the interaction compared with SOCS-1 and -3. We therefore used surface plasmon resonance to examine the interaction of each of the eight SOCS/elonginBC complexes with Cullin5. Experiments were performed using an anti-GST chip to capture GST-Cullin5 (NTD) and then passing over various concentrations (60nM-2μM) of SOCS/elonginBC ternary complexes. All samples fitted well to a 1:1 Langmuir model with the exception of SOCS1/BC which appeared to contain multiple species with different on- and off-rates and had to be excluded from quantitative kinetic analysis. The affinities determined using Biacore were in general agreement with those quantified by ITC, except for SOCS3 which had a 4-fold higher K_D as assessed by biacore. We favour measuring affinity by ITC, as it is less subject to mass-transfer effects and other artifactual limitations, and to use Biacore to determine kinetic constants (k_d and k_a). As is clear from a cursory analysis of the sensorgrams shown in Figure 5, the members of the SOCS family that bind with high affinity to Cullin5 are distinguished from SOCS3 by having a much slower off- rate (10-20 fold slower). Although the off-rate for SOCS1 could not be accurately quantified, the major species also appeared to have a fast off-rate, in keeping with this trend. The slow off-rates associated with the majority of the SOCS family indicate that they form more stable, long-lived, complexes with Cullin5 than do SOCS-1 or -3. Based on the dissociation rate, the half-life of a SOCS3/elonginBC-Cullin5 complex is only 10 seconds compared to 100-200 seconds for the majority of SOCS proteins.

Discussion

The cellular response to cytokine stimulation is temporally regulated with exquisite precision. SOCS proteins play a key role in this regulation by inhibiting the intracellular signalling cascade as part of a negative feedback loop. The molecular details of this inhibition are still relatively unclear but one mechanism potentially shared by all SOCS proteins is to induce the proteasomal degradation of signalling intermediates through the activity of their SOCS box domains. This requires an interaction between the SOCS box and an E3 ubiquitin ligase complex. Previous work has suggested that the specific E3 ligase scaffold relevant for SOCS proteins is Cullin5 but this has not been demonstrated directly for the majority of the SOCS family and in particular it is unclear whether SOCS1 can interact with a Cullin5-based E3 ligase[21].

Our results show that all eight SOCS proteins, including SOCS1, interact with cullin5 but must first recruit elonginBC before doing so. More significantly, the affinity for Cullin5 interaction varies 100-fold across the SOCS family. This highlights two aspects of the interaction that were previously unclear. The first is that pre-formation of a SOCS/elonginBC ternary complex is required before Cullin binding can occur. This imposes a distinct order of binding events

that must occur for the successful formation of a quaternary SOCS/elonginBC/cullin complex. The second and more important observation is that, although structural modelling suggests only minimal direct contact between SOCS and Cullin5, this minimal contact provides the extra affinity that is necessary. In effect, the SOCS box drives the specificity of the interaction, both by directing association with Cullin5, rather than Cullin2[21], and by encoding a two orders-of-magnitude variation in affinity for Cullin5 across the family. This unexpected difference in affinity effectively divides the SOCS family into two classes, those that interact with the E3 ligase complex with high affinity, and SOCS-1 and -3 which do so with significantly lower affinity.

It seems illogical that SOCS-1 and -3, which of all the SOCS family suppress signalling most effectively, should have the lowest affinity for the E3 ligase scaffold, however this may reflect a mechanistic difference in their mode of action. SOCS-1 and SOCS-3 contain a short region upstream of the SH2 domain, the Kinase Inhibitory Region (KIR), which acts by directly inhibiting JAK kinase activity. This does not require the presence of the SOCS box and is independent of the proteasome[7,8,10,12]. Although other SOCS proteins do not contain a KIR, it has been widely suggested that CIS and SOCS2, and potentially other SOCS proteins, may suppress signalling by competing with STATs for their receptor docking sites[27-30], a mechanism which would also be SOCS box independent. However, it is notable that this has never been shown experimentally and in fact it has recently been shown that the STAT5 binding sites on the Growth Hormone Receptor (GHR) are different to those of CIS and SOCS2[31]. Other studies have shown that CIS suppression of STAT activation via EPO and GH is proteasome-dependent[32,28] as is CIS-induced GHR internalisation[33], again implying a purely SOCS box-dependent mechanism. A CIS SH2 domain mutant acted as a dominant negative inhibitor of WT CIS activity, which may be explained by its competition with WT CIS for E3 ligase access[28]. Likewise, SOCS2 with a deleted SOCS box enhanced rather than suppressed growth hormone (GH) signalling which may be explained by its binding to the GH receptor and subsequent blockade of endogenous SOCS2-induced receptor ubiquitination. Other members of the SOCS family also appear to act via SOCS box-dependent mechanisms only. For example, SOCS5 was shown to suppress EGF signalling in a SOCS box-dependent manner *in vitro*[34,35], whilst IRS-1 proteasomal degradation is dependent upon the SOCS7 SOCS box[36,37].

In contrast, with the exception of G-CSF-induced bone marrow cell colony formation, there is no *in vitro* assay in which a role for the SOCS box of SOCS1 or SOCS3 in determining biological activity has been shown[8,28,38]. Consequently the role of the SOCS box of SOCS-1 and -3 had to be assessed *in vivo*, via the generation of SOCS box-null mice. Both SOCS1-box and SOCS3-box knockout mice had a much milder phenotype than the corresponding full SOCS-null animals. SOCS3-box-null mice did not develop inflammatory disease but were hyper-responsive to G-CSF and IL-6 at a level intermediate between WT and conditional SOCS3-null animals[39]. SOCS1 box-null mice show a similar phenomenon: they were hyper-responsive to IFNγ, again to a level intermediate between WT and SOCS1-deleted mice[14]. The much milder phenotype of these animals compared with the full knockouts illustrates that substrate ubiquitination is not the sole or even the primary mechanism of action for SOCS1 and SOCS3.

Our data suggest a correlation between Cullin5 binding affinity and mechanism, with a high affinity for Cullin5 binding reflecting complete reliance on SOCS box-mediated mechanisms for signal suppression. If this correlation holds, then we predict that SOCS-4 and SOCS-6, which also bind tightly to Cullin5, will act through SOCS box-dependent mechanisms only. It is logical that those SOCS proteins which function solely by inducing the ubiquitination of signalling intermediates would require a high affinity interaction with the E3 ligase scaffold. However, given that ancestral SOCS proteins most resemble SOCS5-7, the low affinity of

SOCS-1 and -3 for the E3 ligase scaffold has presumably been selected for over the course of evolution. Of all the members of the SOCS family, it is SOCS-1 and -3 that are primarily responsible for inhibiting signalling from cytokines induced in response to infection. As a consequence of this, knocking out SOCS-1 and -3 genes gives rise to severe inflammatory disease in mice [40,41,42]. It may be that an E3 ligase interaction that is too efficient, or too lengthy, would render them too effective at irreversibly down-regulating this necessary response to infection, thereby not allowing cells to respond to subsequent rounds of stimulation, a result that may be detrimental to the organism. This could be tested *in vivo*, with the generation of SOCS1(IPLN-LPLP) mutant mice.

The affinity of SOCS1 for Cullin5 is low compared to other SOCS proteins yet micromolar affinities are common in biological interactions, especially in intracellular signalling networks where irreversible interactions are usually to be avoided. Many SH2 domain-substrate interactions, including those of the SOCS proteins, are of micromolar affinity for example[24]. The 1μM and 0.1μM affinity of SOCS1 and SOCS3 for Cullin5 allows both proteins to act efficiently as E3 ligase subunits yet has several functional implications. For example, substrate ubiquitination by SOCS-1 and -3 can be predicted to be both less efficient and more susceptible to competition by other SOCS box proteins, especially if Cullin5 levels are limiting (Fig. 6). A study by Hori *et al.*,[45] found that Cullin5 expression was different from other Cullins and was relatively low in all human tissues[43]. The issue of competition is thus pertinent as most cytokines induce a number of different SOCS proteins. For example, expression of SOCS-1, -2, -3 and CIS are all induced by growth hormone and SOCS-1, -2, -3 and -5 are all induced by IL-6[44]. Therefore, by virtue of their weaker binding affinity for Cullin5, SOCS-1 and -3 will not be able to compete with other members of the family for E3 ligase access unless they are either induced earlier or at much higher levels than other SOCS proteins. This adds an extra level of complexity to how cells respond to cytokines.

It has been suggested that, *in vivo*, polyubiquitination of a substrate protein must occur in a single encounter with an E3 ligase, as monoubiquitinated species can be sequestered by ubiquitin binding domain containing proteins or de-ubiquitinated enzymatically[45]. If this is true then the half-life of the substrate-E3 complex will determine the extent of polyubiquitination and the slow off-rates of the majority of the SOCS/Cullin complexes will allow more extensive polyubiquitination than will SOCS-1 and -3. Issues such as these await further studies.

Materials and Methods

Cloning and Expression

DNAs encoding the SOCS box from murine SOCS1-7, CIS were cloned as GST-fusion proteins in the first cloning site of pGEX-4T (human elonginB). This vector contains an internal ribosome entry site preceding the elonginB. The resulting sequences in each case were:

SOCS1: `VRPLQELCRQRIVATVGRENLARIPLNPVLRDYLSSFPFQI`

SOCS2: `APTLQHFCRLAINKCTGTIWGLPLPTRLKDYLEEYKFQV`

SOCS3: `VATLQHLCRKTVNGHLDSYEKVTQLPGPIREFLDQYDAPL`

SOCS4: `PFSLQHICRTVICNCTTYDGIDALPIPSPMKLYLKEYHYKSKVRLLRIDVPEQQ`

SOCS5: `PFSLQYICRAVICRCTTYDGIDGLPLPSMLQDFLKEYHYKQKVRVRWLEREPVKAK`

SOCS6: `VRSLQYLCRFVIRQYTRIDLIQKLPLPNKMKDYLQEKHY`

SOCS7: VKSLQHLCRFRIRQLVRIDHIPDLPLPKPLISYIRKFYYYDPQEEVYLSLKEAQLISKQKQEVEP ST

CIS: ARSLQHLCRLVINRLVADVDCLPLPRRMADYLRQYPFQL

Mutants of the SOCS1 and SOCS3 box were constructed by PCR to yield the following sequences:

$SOCS1_{LPLP}$: VRPLQELCRQRIVATVGRENLARLPLPPVLRDYLSSFPFQI

$SOCS3_{LPLP}$: VATLQHLCRKTVNGHLDSYEKVTQLPLPIREFLDQYDAPL

All proteins cloned in this vector contain five vector-encoded residues after the thrombin cleavage site: SMARQ.

ElonginBC complexes were produced by co-transformation with pBB75 (mouseElonginC$_{17-112}$)[46] into *E. coli* strain BL21(DE3) cells and growing the cells in the presence of ampicillin and kanamycin. Note that mouse and human elonginC sequences are identical. Cells were harvested 8 h after IPTG induction by centrifugation at 6200 *g* at 4 °C for 30 min. SOCS3 lacking the PEST motif [47,48] in complex with elonginBC was produced in the same manner using the same vector. ElonginBC alone was produced using pGEX-4T (elonginB) with elonginC (full-length or 17-112) cloned into site 1. The N-terminal domain (NTD) of murine cullin5 (Met1-Leu385) was expressed as a GST fusion in pGEX-4T. The protein was expressed at room temperature with two point mutations (V341R, L345D) introduced to allow for soluble expression[26]. Cells were resuspended in phosphate-buffered saline (PBS) and lysed by french press. The lysates were centrifuged at 20,000 *g*, and purified using glutathione-Sepharose chromatography. Thrombin was used to remove the GST, followed by size exclusion chromatography on a Superdex 75 or Superdex 200 16/60 column (GE Healthcare) using PBS, 2mM 2-mercaptoethanol (2-ME) as the running buffer.

GST pulldown analysis

The SOCS box domains from SOCS1-7 and CIS were co-expressed with pBB75elonginC in *E. coli* strain BL21(DE3) cells. Following expression, the cells were lysed and GST-Sepharose was used to pull down any proteins in the lysate associated with the GST-SOCS box fragment. After removal of the GST with thrombin, the binding of ternary SOCS-elonginBC complexes to cullin5 was assayed by passing 10 mL of 0.1 mg/mL complex over 0.1mg GST-Cullin (NTD) bound to 0.1 mL glutathione-Sepharose beads. The beads were washed with 50 column volumes of PBS and then dissolved in SDS-containing protein gel loading buffer and the results were visualised by SDS-PAGE followed by Coomassie staining.

NMR spectroscopy

Spectra were recorded at 310 K on a Bruker Avance 500 spectrometer (with cryoprobe) on samples dissolved in 20mM potassium phosphate, 1mM dithiothreitol (DTT). Each elonginBC ternary complex was present at approximately 50 μM. Spectra were processed using Topspin (Bruker AG, Karlsruhe, Germany) and referenced to the H_2O signal at 4.657 ppm (310 K).

Isothermal titration calorimetry—Isothermal calorimetric titrations were performed with a Microcal omega VP-ITC (MicroCal Inc., Northampton, MA). GST-Cullin5 (NTD) and the ternary SOCS box/elonginBC complexes were dialysed against buffer (20 mM potassium phosphate, 100 mM NaCl, 2 mM 2-mercaptoethanol). Experiments were performed at 298 K unless stated otherwise. Typically, 30 ten μl injections of Cullin5 (NTD) were titrated into a 10 μM solution of the SOCSbox/elonginBC ternary complex. The heat of dilution of GST-Cullin5 (NTD) was subtracted from the raw data of the binding experiment. Data were analysed

using the evaluation software, Microcal Origin version 5.0. The binding curve fitted a single-site binding mode and all K_D values were determined from at least duplicate experiments.

Surface Plasmon Resonance (Biosensor) Analysis

GST-Cul5 (NTD) was immobilized onto a Biacore CM5 sensor chip (GE Healthcare) that had been previously coated with anti-GST antibody (GE Healthcare) according to manufacturers instructions. 15 μl of 10 μg/mL GST-Cul5 (sample lane) or GST alone (control lane) was loaded onto the chip resulting in a surface density of ca. 1000 response units. The binding of SOCS/elonginBC ternary complexes to Cul5 was then analysed by diluting the protein in Hepes buffered saline (HBS) containing 0.01% (v/v) Tween-20 and passing it over the chip at 20 μl min^{-1}. The complexes were all analysed at 2, 1, 0.5, 0.25, 0.125, 0.062 μM concentrations. After each sample, the chip was regenerated by the addition of 10mM glycine, pH 2.0. The kinetic data were analysed using the BIAEVALUATION software (GE Healthcare) and fitting to a 1:1 Langmuir binding model. Half-life was calculated using the expression:

$$RU_{(t)}=RU_{0\cdot e^{-k_d t}} \quad (1)$$

where RU is response units and and k_d is the dissociation rate constant. Assuming RU to be linearly proportional to the amount of ligand bound then the half-life of the complex is when $RU_{(t)}$ = ½ RU_0 and equation 1 rearranges to:

$$t_{1/2}= -(\ln\frac{1}{2})/k_d \quad (2)$$

All experiments were performed at least twice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

DTT
: dithiothreitol

GdnHCl
: guanidine hydrochloride

IPTG
: isopropyl-β-D thiogalactopyranoside

ITC
: isothermal titration calorimetry

BME
: β-mercaptoethanol

JAK
: Janus kinase

KIR
: kinase inhibitory region

PBS

phosphate-buffered saline

SOCS
suppressor of cytokine signalling

STAT
signal transduction and activator of transcription

VHL
von-Hippel-Lindau

IFNγ
interferon gamma

IL-6
interleukin 6

G-CSF
granulocyte colony stimulating factor

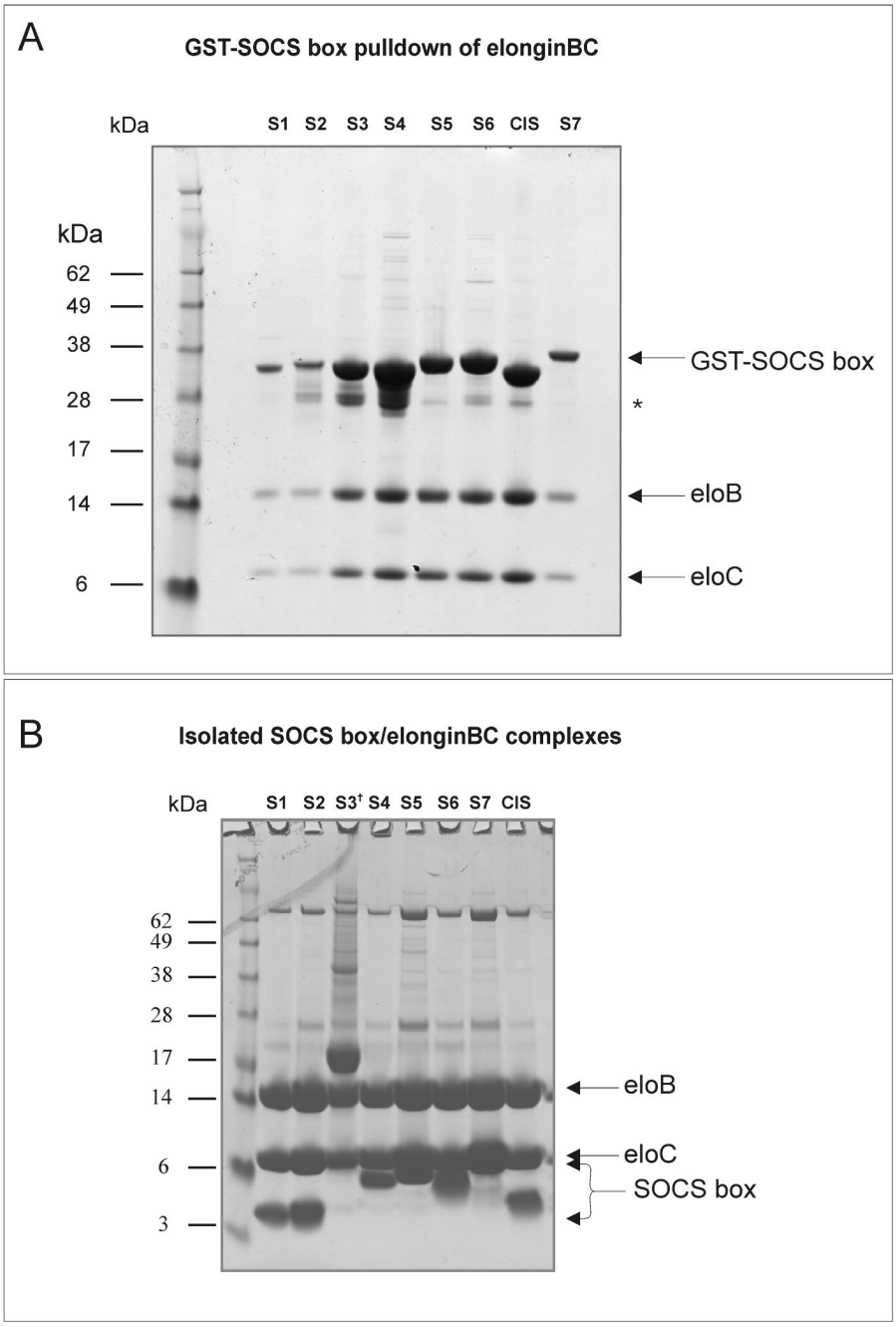

Figure 1. The SOCS box domain from all members of the SOCS family can bind elonginBC

(A) The SOCS box fragments were produced as GST fusion proteins and co-expressed with untagged elonginBC. Glutathione-Sepharose was used to pull down GST labelled proteins present in the cell lysate. As shown, the SOCS box domain from all eight members of the SOCS family could pull down elonginBC. Taking into account the different levels of SOCS expression there was no obvious difference in affinities among different members of the SOCS family. The asterisk indicates unavoidable protein degradation. (B) GST pulldowns such as those shown in (A) were digested with thrombin to remove the GST, then purified by gel filtration and concentrated to 10 mg/mL. In this case full-length SOCS3 was used as a positive control (lane S3†), as the association of the SOCS3 box with elonginBC has already been

described[22]. The SOCS box of SOCS7 co-migrates with elonginC and so is not resolved by SDS-PAGE, however its presence was verified by mass-spectrometry (data not shown) and can be observed by NMR (Figure S1).

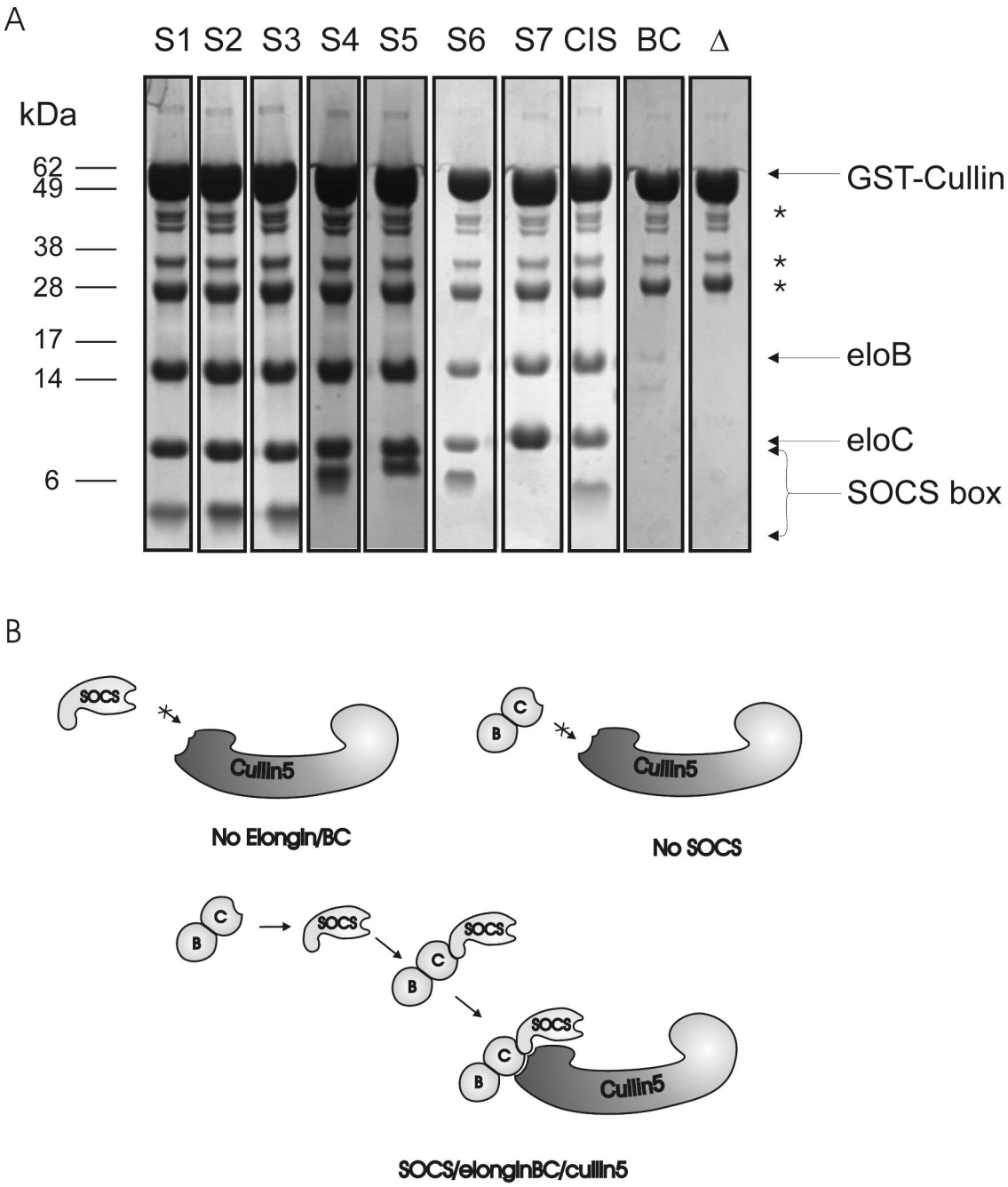

Figure 2. All members of the SOCS family, when in complex with elonginBC, bind cullin 5

(A) Ternary SOCS box/elonginBC complexes were incubated with glutathione Sepharose beads bound to a GST fusion of the N-terminal domain of cullin5. The SOCS box domains of all SOCS family members (S1-7, CIS), when bound to elonginBC, were shown to interact with cullin5. ElonginBC alone (BC) did not interact with cullin5. A fragment of the SOCS3 SOCS box, lacking the predicted cullin-binding motif (ΔC28) was used as a negative control (Δ). Asterisks mark GST-cullin5 degradation products, which are consistent across all experiments and were verified by mass spectrometry. Note that the SOCS7 box domain co-migrates with elonginC on SDS-PAGE. (B) Schematic view of our findings. Cullin5 binding requires a

ternary SOCS/elonginB/elonginC complex. Neither elonginBC alone, nor a SOCS protein alone associate with the E3 ligase scaffold with measurable affinity.

Protein	K_D (nM)	ΔH (kcal/mol)	
SOCS1/BC	1000 ± 80	-4.0 ± 0.5	Class 1 (K_D: 10^{-6}-10^{-7} M)
SOCS3/BC*	105 ± 10	-3.7 ± 0.5	
SOCS2/BC	9.5 ± 3	-7.8 ± 2.0	Class II (K_D~10^{-8} M)
SOCS4/BC	10 ± 2	-6.1 ± 2.0	
SOCS5/BC	7 ± 3	-3.5 ± 0.3	
SOCS6/BC	23 ± 10	-4.3 ± 0.6	
SOCS7/BC	40 ± 20	-5.4 ± 1.5	
CIS/BC	19 ± 6	-4.7 ± 1.0	
BC*	no binding (> 10^5)	N/A	
SOCS3	no binding (> 10^5)	N/A	

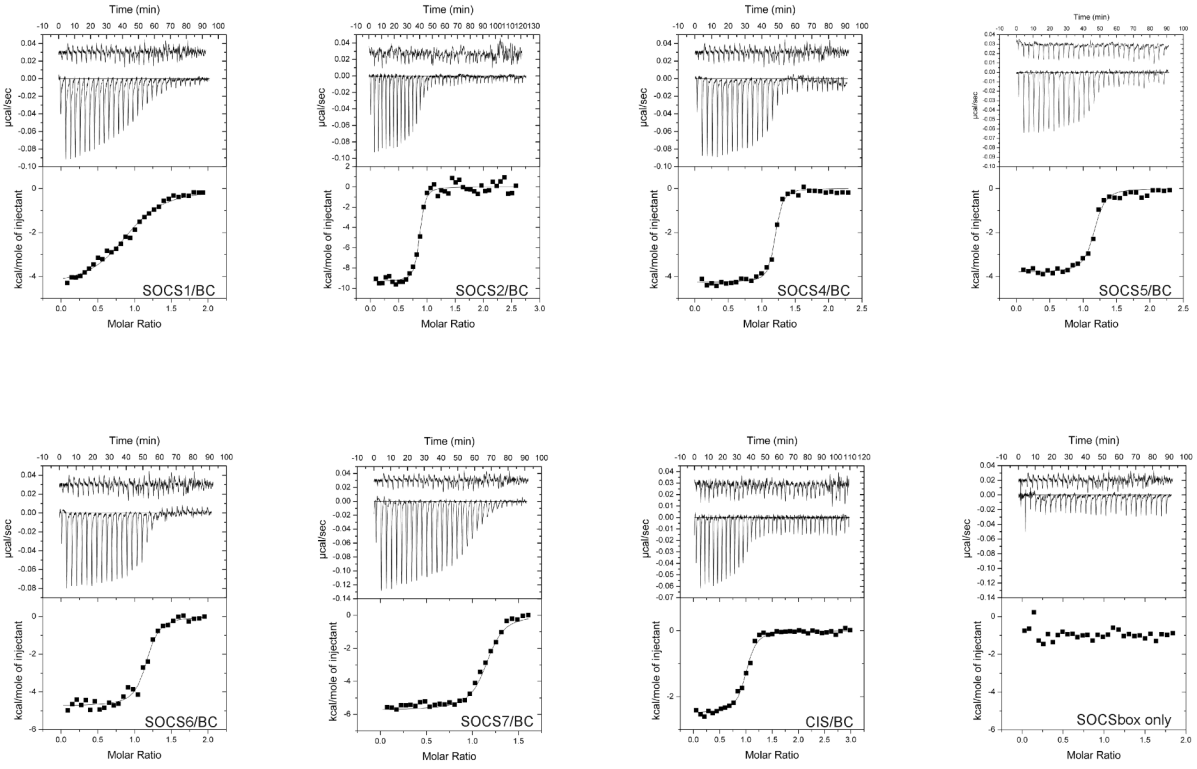

Figure 3. The affinity for Cullin5 describes two sub-classes of SOCS protein

ITC analysis of the SOCS box/elonginBC-cullin5 interaction was used to measure the affinity of each SOCS box for Cullin5. (A) Tabular view of the results showing those proteins that interacted relatively weakly with Cullin5 (Class I), those that interacted strongly (K_D ~ 10 nM, Class II) and controls that did not interact at all. The values listed are the mean +/- S.D from two independent experiments using individually prepared batches of protein. The data for SOCS3/BC and elonginBC alone are from Babon *et al*[22] (asterisks). (B) ITC data. 80 μM GST-cullin5 (NTD) was titrated into 10 μM SOCS box/elonginBC complex. The titration curves all fitted well to a single-site model with ~ 1:1 stoichiometry within experimental error.

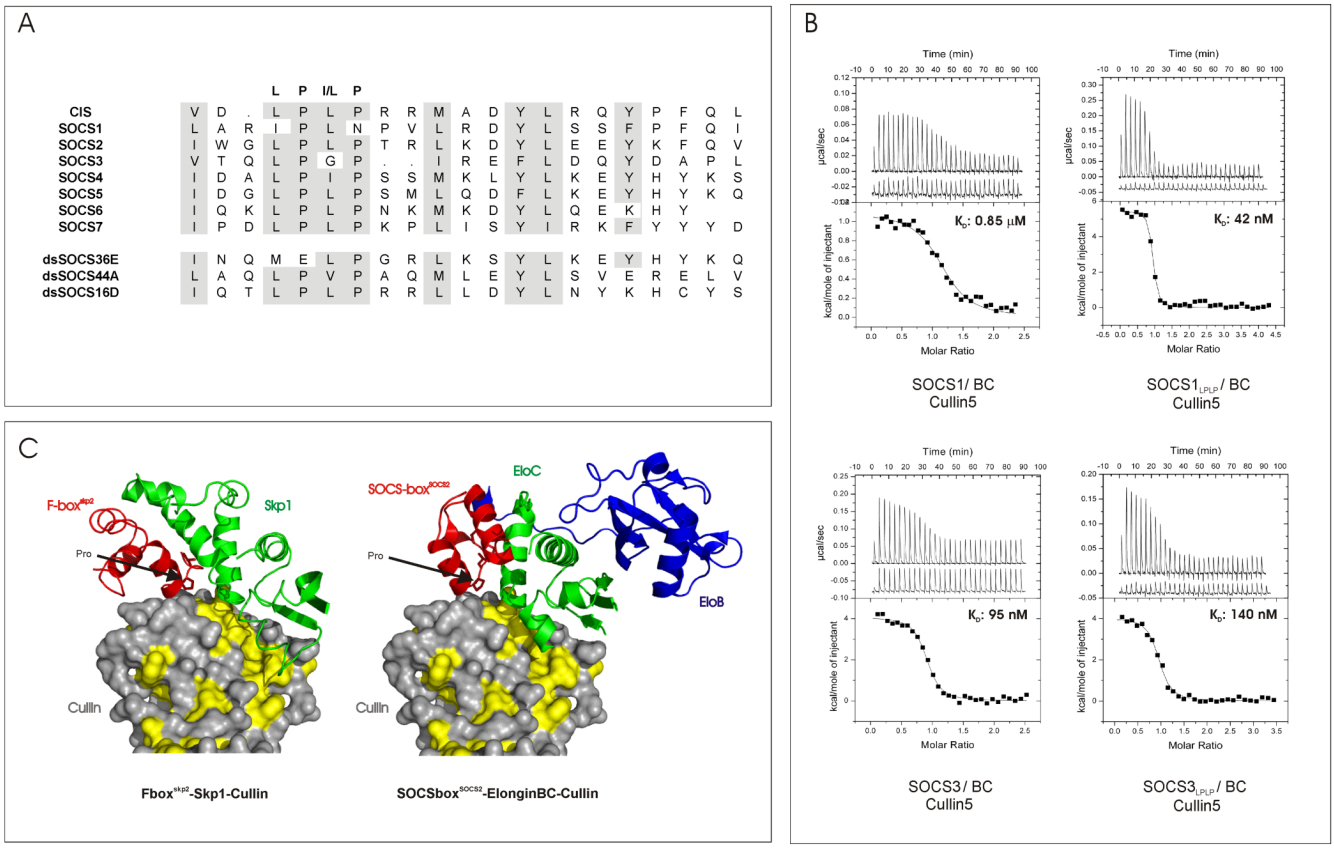

Figure 4. Analyses of the SOCS/elonginBC interaction

(A) Sequence comparison of the SOCS box from mouse CIS, SOCS1-7 and the three *Drosophila* SOCS proteins with the Cullin5-binding motif (LPLP) and other conserved residues shown highlighted in grey. (B) Mutating SOCS-1 and SOCS-3 to generate the canonical Cullin5 binding motif (LPLP) restores the affinity of Cullin5 for SOCS1 but not SOCS3. ITC analysis of the interaction between SOCS1(LPLP) and SOCS3 (LPLP) mutants with Cullin5. Because of a decreased enthalpy of interaction for both mutants the signal to noise ratio was low at 25°C (SOCS1(LPLP)=1.5 kcal/mol, Figure S2). The SOCS3(LPLP) interaction was too low to be measured, data not shown) so the reactions were repeated at 5° C where the interaction is endothermic in order to yield more accurate results. SOCS3 wild-type and mutant are shown in the lower two panels performed at 5°C. The titration curves all fitted well to a single-site model with ~ 1:1 stoichiometry within experimental error. (C) Modelling of the SOCS2 box/elonginBC (PDB 2C9W) interaction with Cullin5 (right) using the Fbox-Skp2-Cullin1 structure (left) as a template (PDB 1LDK) is shown. The surface of Cullin is shown (grey) with hydrophobic residues in yellow and the ligands are represented as ribbon structures.

	SOCS1	SOCS2	SOCS3	SOCS4	SOCS5	SOCS6	SOCS7	CIS	SOCS1$_{LPLP}$
k_a ($M^{-1}s^{-1}$)	N/A	1.45 x 10^5	1.75 x 10^5	2.75 x 10^5	3.06 x 10^5	7.50 x 10^5	2.99 x 10^5	1.56 x 10^5	2.20 x 10^5
k_d (s^{-1})	N/A	6.81 x 10^{-3}	0.0692	3.47 x 10^{-3}	5.42 x 10^{-3}	5.50 x 10^{-3}	5.80 x 10^{-3}	6.70 x 10^{-3}	15.3 x 10^{-3}
K_D	N/A	47 nM	397 nM	13 nM	18 nM	8 nM	20 nM	43 nM	69 nM

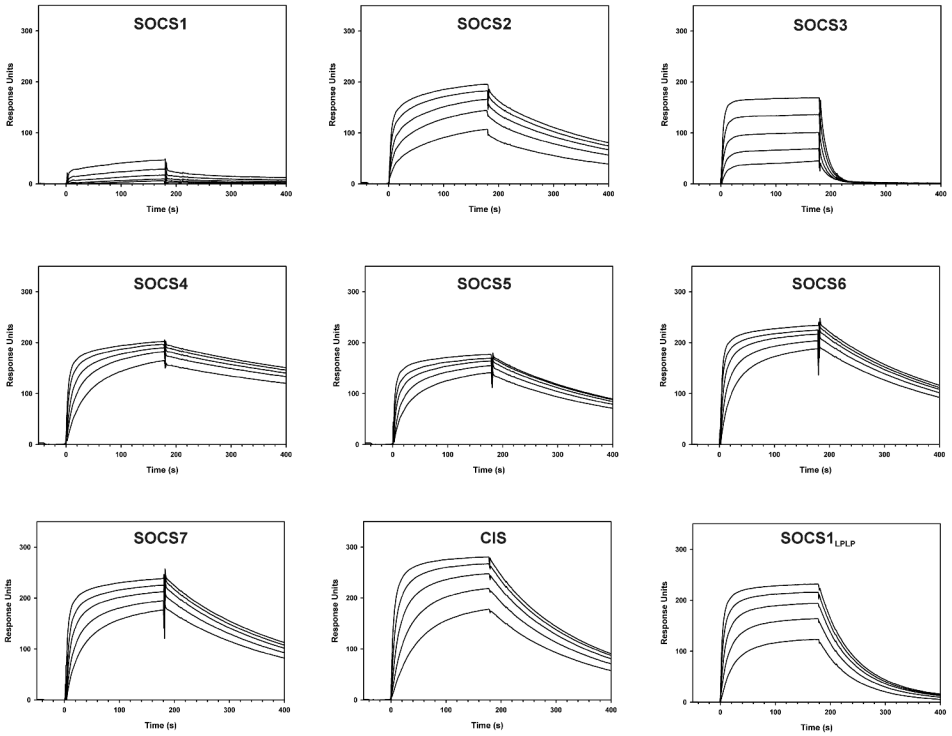

Figure 5. Surface plasmon resonance (Biacore) analyses of the SOCS/elonginBC-Cullin5 interaction

Sensorgrams of SOCS/elonginBC complexes binding to GST-Cullin5. Anti-GST antibody was covalently attached to a Biacore chip and used to capture GST-Cullin5 (NTD). Ternary SOCS/elonginBC complexes were then passed over the chip at 1, 0.5, 0.25, 0.125, 0.061 μM concentration (upper to lower curves in each panel). The curves were used to perform a global fit of the association and dissociation rates and the dissociation constant, the values are given below. All interactions could be fitted using a simple 1:1 Langmuir model with the exception of the SOCS1/elonginBC-Cullin5 interaction (*top left*) which appeared to have multiple binding components and was hence excluded from quantitation. All sensorgrams are plotted on the same scale for ease of comparison.

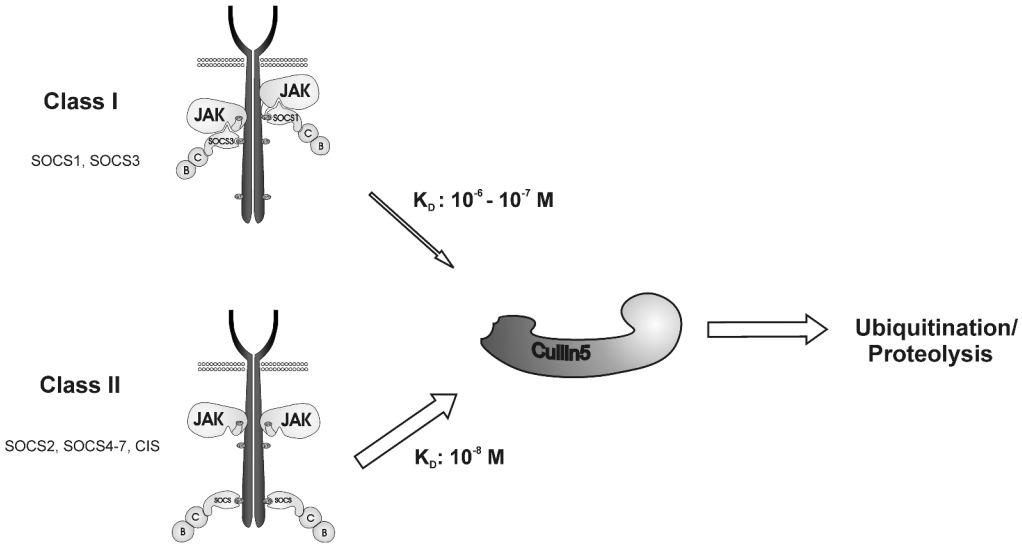

Figure 6. Schematic view of the division in the SOCS family based on mechanism and Cullin5 binding affinity

SOCS-1 and SOCS-3 define a separate class of SOCS proteins (termed Class I) based on their relatively low affinity for Cullin5 and the fact that they can interfere with signalling by associating with and inhibiting JAK. These two SOCS proteins are the most active at suppressing signalling and appear to be the most important regulators of the cytokine response to infection and inflammation. They are at least partially active in the absence of the SOCS box domain. The remaining SOCS proteins (class II) all bind tightly to Cullin5 and we suggest that the SOCS box is indispensable for their activity. Based on their higher affinity for the E3 ligase scaffold, class II SOCS proteins will be able to out-compete class I for cellular ubiquitination machinery. Both Classes of SOCS protein have to bind to elonginBC before the E3 ligase interaction will take place.