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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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The SOCS (suppressors of cytokine signalling) family of proteins inhibits 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. In this study, we dissected this interaction *in vitro* using purified components. We found 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 2 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 the rest of the family. The remaining six SOCS proteins all bound Cullin5 with high affinity (K_d of ~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 SOCS-3 have been shown to suppress signalling using both SOCS box-dependent and SOCS box-independent mechanisms. This is not the case with the other six SOCS proteins, and our data imply the existence of two distinct subclasses of SOCS proteins with a high affinity for Cullin5, the E3 ligase scaffold, possibly reflecting complete dependence upon ubiquitination for suppression of cytokine signalling.

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Keywords: SOCS; cytokine signalling; ubiquitin ligase; elongin; Cullin

Introduction

SOCS (suppressors of cytokine signalling) 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 reorientation. This allows Janus Kinases (JAKs), associated with the intracellular domains of the receptor, to initiate a cascade of tyrosine phosphorylation events that eventually result in STAT (signal transducers and activators of transcription)-mediated up-regulation 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: SOCS-1 to SOCS-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 (NTD) of varying lengths

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Abbreviations used: CIS, cytokine-inducible SH2 domain-containing protein; GH, growth hormone; GST, glutathione *S*-transferase; ITC, isothermal titration calorimetry; JAK, Janus kinase; NTD, N-terminal domain; PBS, phosphate-buffered saline; SOCS, suppressors of cytokine signalling; STAT, signal transducers and activators of transcription; VHL, von Hippel–Lindau; WT, wild type.

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 degradation.[19] E3 ubiquitin ligases, in the presence of ubiquitin-activating and ubiquitin-conjugating enzymes (E1 and E2, respectively), catalyse the covalent addition of ubiquitin to lysine side chains on target proteins.[20] Polyubiquitination of proteins then leads to their recognition and degradation by the proteasome. According to this model, SOCS proteins, such as 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 domain and NTD. As all eight SOCS proteins contain a central SH2 domain, tyrosine-phosphorylated signalling intermediates, such as phospho-JAK, phospho-STAT and even phosphorylated receptors, are potential substrates.

In support of this hypothesis, SOCS-3 was shown to interact with Cullin5 and Rbx2.[21] Cullins are a family of scaffold proteins for the Cullin–RING type of E3 ligases. The C-terminal domain of Cullin5 associates with Rbx2, a RING finger protein that acts as a docking site for the E2 enzyme, while the NTD 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 SOCS-1 as no interaction with Cullin5 or Cullin2 was detected using endogenous protein,[21] although a SOCS-1–Cullin2 interaction has been implied using overexpression systems.[18] In addition, SOCS-1 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 SOCS-1, ubiquitin ligase activity has not been demonstrated for SOCS-2–SOCS-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 SOCS-1, 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. SOCS-1 and SOCS-3 bind relatively weakly to Cullin5, while the rest of the family associates more tightly with a dissociation constant of $\sim 10^{-8}$ 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 SOCS-1 and SOCS-3. 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 a 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 utilise a Cullin5-based E3 ligase, we undertook *in vitro* binding studies using purified recombinant proteins. This approach has the advantages over co-precipitation studies of allowing affinity measurements and 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 SOCS-3 acts as an independent domain that interacts with Cullin5 with affinity and enthalpy identical with those for 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 SOCS-1–SOCS-7 and CIS were cloned, co-expressed with elonginB and elonginC in *Escherichia coli* and purified. The glutathione *S*-transferase (GST) pull-down data in Fig. 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.