SOCS proteins and cytokine signalling:

The many faces of the SOCS box

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dedicated to Jacques Piessevaux
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**Abbreviations**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>amino acids</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>β_c</td>
<td>common beta chain</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>CIS</td>
<td>cytokine inducible SH2 containing protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNTF</td>
<td>ciliary neurotrophic factor</td>
</tr>
<tr>
<td>CRH</td>
<td>cytokine receptor homology</td>
</tr>
<tr>
<td>db</td>
<td>mouse diabetes gene, coding for the leptin receptor</td>
</tr>
<tr>
<td>DIO</td>
<td>diet-induced obesity</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Epo</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FNIII</td>
<td>fibronectin type III domain</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>γ_c</td>
<td>common gamma chain</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>GGS</td>
<td>Gly-Gly-Ser</td>
</tr>
<tr>
<td>GHR</td>
<td>growth hormone receptor</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth receptor bound protein 2</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia induced factor-1 α</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>inositoltrisphosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>ISRE</td>
<td>interferon stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>JAK homology</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino-terminal kinase</td>
</tr>
</tbody>
</table>
KIR  kinase inhibitory region
LR  leptin receptor
LIF  leukaemia inhibitory factor
LPS  lipopolysaccharides
MAPK  mitogen activated protein kinase
MAPPIT mammalian protein-protein interaction trap
MS  mass spectrometry
NES  nuclear export signal
NF-κB nuclear factor κB
NGF  nerve growth factor
NK  natural killer cell
NLS  nuclear localisation signal
ob  mouse obese gene, coding for leptin
OSM  oncostatin M
PDE3B phosphodiesterase 3B
PH  pleckstrin-homology
PI3K phosphoinositol 3-kinase
PIAS protein inhibitor of activated STAT
PIP2 phosphatidylinositolbiphosphate
PKC phosphokinase C molecules
PLCγ phospholipase C gamma
PRL prolactin
PTB phosphotyrosine-binding
PTP 1B protein tyrosine phosphatase 1B
Φ hydrophobic
SH2 Src homology 2
SHP SH2 domain containing phosphatase
SOCS suppressors of cytokine signalling
STAT signal transducer and activator of transcription
SUMO small Ub-related modifier
TAP tandem affinity purification
Th T helper subtype
TLR toll-like receptors
TNF tumour necrosis factor
Ub ubiquitin
VEGF vascular endothelial growth factor
VHL Von Hippel Lindau
Summary

SOCS proteins are regulators of various signal transduction cascades that are essential to normal physiology. The importance of a strict control of signalling is underscored by the contribution of aberrant SOCS functions in several pathologies. So far, eight SOCS members have been identified (CIS and SOCS1-7) and they are all characterised by common structural motifs: a central SH2 domain and a C-terminal SOCS box. The SH2 domain defines substrate selectivity, while the SOCS box domain mediates the interaction of SOCS proteins with the Elongin B/C complex, linking them to the ubiquitin/proteasome degradation system. Upon stimulation, SOCS proteins are recruited to activated receptors, where they suppress signalling by different mechanisms including targeting of the receptor complex for proteasomal degradation.

The first part of this thesis focuses on the role of SOCS proteins in leptin receptor (LR) signalling. Hypothalamic leptin responses play a central role in body weight regulation. In addition, leptin also has direct effects on peripheral cell types involved in regulation of diverse body functions including immune response, haematopoiesis and reproduction. Since alterations in normal leptin action have severe pathological implications, it is important to understand the mechanisms that regulate leptin signalling. Previous studies have demonstrated the important role of SOCS3 in leptin physiology. Using MAPPIT, a cytokine receptor-based two-hybrid method operating in intact mammalian cells, we showed that CIS and SOCS2 can also bind to the LR and we further characterised the binding mode and functionality of these interactions. Next, we studied the signalling events of the LR in haematopoiesis. Therefore, we designed a novel MAPPIT variant, βc-MAPPIT, which allows analysis in a haematopoietic environment. We identified several reported as well as novel interactions with the LR, including binding of SOCS proteins.

The SOCS box domain of SOCS molecules was known to be involved in targeting associated proteins for degradation and in SOCS protein stability. In the second part of the thesis, we reported novel roles for this domain. First, the SOCS box was demonstrated to be implicated in cross-modulation between SOCS proteins. This way, some SOCS members can interfere with the inhibitory functions of other SOCS proteins by targeting them for proteasomal degradation. This cross-regulatory mechanism may be involved in restoring the basal cellular responsiveness for subsequent stimulation. Secondly, we showed that the SOCS box can regulate substrate binding. Receptor interaction and functionality of CIS were found to crucially require Elongin B/C recruitment to the SOCS box. This Elongin B/C-dependency appears to be unique for CIS and represents a novel regulatory mechanism by which the SOCS box may form an on/off switch acting on the SH2 domain. Together, our findings indicate multiple roles for the SOCS box.
Samenvatting

De familie van SOCS eiwitten zijn essentiële regulatoren in verscheidene signalisatie cascades. Het belang van een strikte controle van de signalisatie wordt benadrukt door het voorkomen van aberrante SOCS functies die bijdragen tot verschillende ziektepatronen. Tot op heden zijn acht SOCS proteïnen geïdentificeerd (CIS en SOCS1-7) en ze worden gekenmerkt door gemeenschappelijke structurele motieven: een centraal SH2 domein en een eindstandige SOCS box. Het SH2 domein bepaalt de substraat selectiviteit en het SOCS box domein staat in voor de interactie met het Elongine B/C complex dat een link vormt naar het ubiquitine/proteasoom afbraaksysteem. Na stimulatie worden de SOCS eiwitten gerecruteerd naar de geactiveerde receptor waar ze door verschillende mechanismen de signaalweg zullen onderdrukken.

In het eerste deel van dit werk gaan we dieper in op de functies van de SOCS eiwitten in leptine signalisatie. Leptine heeft een centrale rol in de regulatie van het lichaamsgewicht. Bovendien heeft leptine ook effecten op bepaalde perifere weefsels die ondermeer betrokken zijn bij immuniteit, haematopoiesis en vruchtbaarheid. Aangezien ongepaste wijzigingen in leptine responsen betrokken zijn bij verscheidene ziektes, is het van groot belang om de mechanismen die de leptine signalweg regelen te ontrafelen. Vorige studies hebben een belangrijke rol voor SOCS3 in leptine fysiologie aangetoond. Gebruikmakend van MAPPIT, een twee-hybride methode die werkzaam is in intacte zoogdiercellen, tonen wij aan dat CIS en SOCS2 ook met de leptine receptor (LR) kunnen binden. Vervolgens werden de signalisatie cascades van de LR in hematopoëse bestudeerd. Daarvoor ontwierpen we een nieuwe MAPPIT variant, βc-MAPPIT, die analyse in haematopoëtische cellen toelaat. Hiermee konden we zowel gekende als nieuwe interacties met de LR identificeren waaronder ook die met SOCS eiwitten.

De SOCS box was gekend om zijn sleutelfunctie in het regelen van de SOCS stabiliteit en het merken van geassocieerde eiwitten voor proteasomale afbraak. In het tweede deel van deze thesis bewijzen we nieuwe functies voor dit domein. Eerst tonen we aan dat de SOCS box een functie heeft in de onderlinge modulatie van SOCS eiwitten. Op deze manier kunnen sommige SOCS leden interferen met de inhiberende werking van andere SOCS eiwitten. Dit interfererend mechanisme zou van belang kunnen zijn in het herstellen van de cellulaire gevoeligheid. Vervolgens tonen we aan dat de SOCS box substraat binding kan regelen. Receptor interactie en functionaliteit van CIS bleken immers Elongine B/C recrutering aan de SOCS box te vereisen. Deze Elongine B/C afhankelijkheid is uniek voor CIS en vertegenwoordigt een nieuw regulatorisch mechanisme waarmee de SOCS box de functies van het SH2 domein kan beïnvloeden. Samengevat wijzen onze bevindingen op veelvoudige functies voor de SOCS box.
Résumé

Les protéines SOCS forment une famille de régulateurs impliquée dans diverses cascades de signalisation. Le disfonctionnement des SOCS contribue au développement de plusieurs maladies. Il est donc nécessaire de contrôler la signalisation rigoureusement afin d’éviter cela. Jusqu’à présent, huit membres SOCS on été identifiés (CIS et SOCS1-7). Ils sont caractérisés par des motifs communs : un domaine SH2 au centre et le SOCS box à la fin. Le domaine SH2 définit l’interaction avec le substrat et le SOCS box maintient l’association avec le complexe des Elongines B et C, reliant les SOCS au système de dégradation par le protéasome. Après stimulation, les SOCS sont recrutées vers les récepteurs activés et suppriment la signalisation par différents mécanismes.

Dans la première partie de cette thèse nous approfondissons les fonctions des protéines SOCS dans la signalisation de la leptine. Non seulement la leptine joue un rôle central dans le réglage du poids corporel mais elle a également des effets directs sur les tissus périphériques où elle module diverses fonctions comme l’immunité, l’hématopoïèse et la reproduction. Il est nécessaire d’obtenir une meilleure compréhension des mécanismes qui régellent la signalisation de la leptine car un dérèglement mènerait vers des implications pathologiques. Plusieurs études démontrent le rôle crucial de SOCS3 dans la physiologie de la leptine. En utilisant MAPPIT, une stratégie deux-hybride opérant dans des cellules mammifères intactes, nous prouvons que CIS et SOCS2 peuvent également s’associer au récepteur de la leptine. Nous avons également étudié les cascades de signalisation du récepteur de la leptin dans l’hématopoïèse. Pour cela, nous avons conçu une variante de la technique MAPPIT, βc-MAPPIT, qui permet des études de cellules hématopoïétiques. Nous avons relevé plusieurs interactions connues mais également des nouvelles y compris celles des SOCS.

Le SOCS box est connu pour son implication dans la modulation de la stabilité des SOCS, ainsi que pour son rôle dans la dégradation protéasomale de protéines associées. Dans la seconde partie de cette thèse, nous identifions de nouveaux rôles pour ce domaine. Nous rapportons que le SOCS box est impliqué dans la modulation réciproque entre protéines SOCS. Suite à cela certains membres peuvent interférer avec les fonctions suppressives d’autres SOCS en les marquant pour la destruction protéasomale. Il est probable que le mécanisme de modulation réciproque entre SOCS soit impliqué dans la reconstitution des réponses cellulaires à de nouvelles stimulations. Nous démontrerons ensuite que le domaine SOCS box est capable de régler l’association de SOCS avec leur substrat. Il s’avère que l’interaction avec le récepteur et la fonctionnalité de CIS nécessitent le recrutement du complexe Elongin B/C au SOCS box. Cette dépendance est unique pour CIS et représente un nouveau mécanisme régulateur par lequel le SOCS box peut contrôler les interactions du domaine SH2. En conclusion, nos résultats démontrent des rôles variés et complexes pour le SOCS box.
Part I: General Introduction
CHAPTER 1: Cytokine receptors and signal transduction

The complexity of multicellular organisms is made possible by the evolution of systems enabling cells to communicate and consequently respond to distinct cues. Cytokines and their receptors represent one such system that plays a key role in cell proliferation, differentiation, migration and apoptosis. These biological activities are critical to processes as diverse as haematopoiesis, immune responses, growth and embryonic development.

I. Cytokines

Cytokines are defined as a highly heterogeneous group of small and secreted messenger proteins, some of which remain cell bound, that are involved in intercellular communication in multicellular organisms. Two crucial features that characterize the cytokine network are their pleiotropy and redundancy. Pleiotropy indicates that one cytokine can provoke a broad range of responses depending on the cell type or the differentiation stage. This way, cytokines can orchestrate a coordinated response of different cellular processes. Redundancy implies that different cytokines can exert similar biological activities. In this way, important cellular mechanisms are preserved by a back-up mechanism, in which one cytokine can compensate for the loss of another. These compensatory mechanisms can be explained in part by the common use of certain receptor chains and signalling molecules by the different cytokines.

A cytokines’ sphere of action is limited: they are secreted in very low amounts, and producer and responder cells are often physically located close to each other (thereby cytokines differ from classical hormones). Dispersal of some cytokines is also limited by binding to the extracellular matrix and soluble cytokine receptors that bind and inhibit the biological actions of cytokines.
Originally, cytokines were classified based on their biological responses. But given the apparent functional pleiotropy and low sequence homology, current cytokine classification relies on structural similarities. According to the Cytokine Web (http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/index.html) different fold families can be distinguished (table 1).

Table 1: Classification of the cytokines based on similarity in protein folding

<table>
<thead>
<tr>
<th>α-helical structure</th>
<th>long chain 4-α-helix bundle superfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>interleukin-6 (IL-6), growth hormone (GH), Leptin, erythropoietin (Epo), prolactin (PRL), granulocyte-colony stimulating factor (G-CSF), myelomonocytic growth factor, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cholinergic differentiation factor (CDF)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>short chain 4-α-helix bundle superfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2, IL-3, IL-4, IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>dimeric 4-α-helix bundle superfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>interferon γ (IFNγ), interferon β (IFNβ), IL-10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>β-sheet structure</th>
<th>B-Trefoil</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1-α, IL1-β, fibroblast growth factor (FGF)</td>
<td></td>
</tr>
</tbody>
</table>

| β-sandwich | tumour necrosis factor α (TNFα), tumour necrosis factor β (TNFβ) |

| β-EGF-like | transforming growth factor α (TGF-α) |

| β-Cystine knot dimerization domains | gonadotropin, nerve growth factor (NGF), platelet-derived growth factor (PDGF), transforming growth factor-β2 (TGF-β2) |

| α/β-structure | IL-8, platelet factor 4 (PF-4), macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, melanoma growth stimulating activity (MGSA) |
Cytokines studied in this work, including leptin, GH, Epo and IFNβ, belong to the 4-α-helical bundle cytokine family. These share a typical fold consisting of 4 α-helices arranged in an up-up-down-down orientation, linked together with 2 loops. The 'long chain' subgroup has long α-helices and their loops contain additional helices, while the subgroup of the 'short chain' cytokines has shorter α-helices and their loops comprise two short antiparallel β-strands. Cytokines of the third subgroup form dimeric structures (figure 1).

---

**Figure 1: Structures of 4 α-helix bundle cytokines**
GH, a long chain 4-α-helical bundle cytokine (left). GM-CSF, a short chain 4-α-helical bundle cytokine (right). IFNγ, a dimeric 4-α-helical bundle cytokine (under) (adapted from 'The Cytokine Web')

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Chapter 1: Cytokine receptors and signal transduction
II. Cytokine receptors

Since cytokines are unable to cross the cellular membrane themselves and have no intrinsic enzymatic activity, they require specific transmembrane receptor proteins to transmit their signal to the inside of cells. Based on (predicted) secondary and tertiary structure similarities, cytokine receptors can be divided into 4 classes (table 2). In general, there is a clear correlation between the structural class of the cytokine ligands and their receptors.

Table 2: Classification of the cytokine receptors based on structure similarities.

<table>
<thead>
<tr>
<th>class</th>
<th>name</th>
<th>characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>haematopoietin/interferon receptors</td>
<td>Ig-like and FNIII-like domains in the extracellular part unstructured intracellular part</td>
</tr>
<tr>
<td>II.</td>
<td>TNF/IL-1 receptors</td>
<td>repeat of 6 Cys in the extracellular part often presence of a death domain (induction of apoptosis) in the intracellular part</td>
</tr>
<tr>
<td>III.</td>
<td>receptor kinases</td>
<td>intrinsic kinase activity: phosphorylation of Tyr (e.g. insulin receptor) or Ser/Thr (e.g. TGFβR)</td>
</tr>
<tr>
<td>IV.</td>
<td>serpentine receptors</td>
<td>cross the cellular membrane seven times Ser/Thr residues in intracellular part, acting as phosphorylation sites for receptor regulation</td>
</tr>
</tbody>
</table>

Cytokine receptors examined in this thesis are part of the class I receptor family. Members of this receptor class are characterized by a conserved extracellular module, known as the cytokine receptor homology domain (CRH), along with a range of other structural modules including immunoglobulin (Ig)-like and fibronectin type III (FNIII)-like domains, a transmembrane and intracellular domains (Bazan, 1990; Kishimoto et al., 1994). The CRH domains consist of two homologous barrel-like structures of 7 β strands resembling the FNIII fold. The cytoplasmatic domains of the members of this receptor class are more heterogeneous. Unstructured intracellular part (see table 2) points to the lack of structural information, either by NMR or by crystallography studies, on the cytoplasmatic domains of the class I
cytokine receptors. This may well reflect the absence of clearly structured subdomains. Moreover, beside the membrane-proximal box1 and box2 motifs and conserved tyrosine residues there are no well-conserved sequences or elements within the cytosolic tails of these receptors. Haematopoietin receptors are divided into two groups which have divergent CRHs (Bazan, 1990).

The type I receptors are characterized by two conserved disulfide bonds and a canonical Trp-Ser-X-Trp-Ser (WSXWS) motif in their CRH (Bazan, 1990). Some members are composed of a single receptor subunit, like the GH receptor (GHR), Epo receptor (EpoR) or PRL receptor (PRLR) and are assembled in homomeric structures (figure 2). However, the majority of type I cytokine receptors consists of different receptor chains and fall into three major families based on usage of shared receptor chains. These receptor complexes combine a ligand-specific subunit with a shared signal transducing chain. Such shared receptor subunits are (i) the gamma
common receptor (γc) chain, predominantly utilized by the IL-2 subfamily (IL-2, IL-4, IL-7 and others) (Ozaki and Leonard, 2002); (ii) the beta common (βc) chain, used by the IL-3 subfamily (IL-3, IL-5 and GM-CSF) (Boulay et al., 2003; Ozaki and Leonard, 2002) and (iii) the glycoprotein 130 (gp130) chain, shared by the IL-6 subfamily (IL-6, IL-11, LIF and others). Type II receptors have also two pairs of cysteines but with a different arrangement and they lack the WSXWS motif (Bazan, 1990). The common use of receptor chains results in 10 receptor complexes formed from a pool of 12 type II receptor chains (Kotenko and Langer, 2004; Langer et al., 2004). Type II receptor members are activated by the IFN and IL-10 family and they are primarily involved in antiviral and inflammatory responses (Renauld, 2003)

III. The JAK-STAT pathway

Members of the class I cytokine receptor family have no intrinsic kinase activity and therefore typically depend on intracellular associated janus kinases (JAKs) to trigger signal transduction. A schematic outline of the JAK-STAT pathway is shown in figure 3. Binding of a cytokine to its receptor complex (step 1) will induce clustering and/or reorganization of the receptor chains in such a way that the associated JAKs will be brought in close proximity, allowing them to activate each other by cross-phosphorylation (step 2). These activated kinases will then phosphorylate tyrosine residues in the cytoplasmatic portion of the receptor (step 3). These phosphotyrosines provide docking sites for various signalling proteins containing a phosphotyrosine-binding domain, like a Src homology 2 (SH2) domain or the less common phosphotyrosine-binding domain (PTB). Signal transducers and activators of transcription (STAT) proteins dock to these phosphotyrosine containing motifs. The STAT molecules then become phosphorylated themselves by the JAKs upon receptor association (step 4). Subsequently, the activated STATs dissociate from the receptor (step 5) and migrate to the nucleus as dimers (step 6), where they act as transcription factors to initiate transcription of specific target genes (Ihle et al., 1994; Kisseleva et al., 2002; O'Shea et al., 2002).
A. *The JAK family*

This kinase family is named after Janus, the Roman god of gates and doors, beginnings and endings. Therefore Janus is represented with a double-faced head, each looking in an opposite direction. This is reminiscent of the distinctive feature of JAK structure: their kinase and pseudokinase domain. In mammals, the JAK family comprises four members: JAK1-3 and Tyk2. JAK1, JAK2 and Tyk2 are expressed ubiquitously, while the expression of JAK3 is restricted to cells of haematopoietic origin (Leonard and O'Shea, 1998).

*Structure of JAKs*

JAKs are relatively large proteins of approximately one thousand amino acids (AA). Comparison of JAK sequences reveals seven regions of high similarity, called JAK homology (JH) domains 1 to 7 (for an overview see (Leonard and O'Shea, 1998) (figure 4). The C-terminal JH1 and JH2 domains encode respectively a kinase and pseudokinase domain. Although this latter domain contains structural features of a...
tyrosine kinase, it is devoid of any catalytic activity. Reports suggest that this domain modulates the catalytic activity of the kinase domain (Saharinen et al., 2000; Velazquez et al., 1995; Yeh et al., 2000). The JH1 kinase domain behaves like a classical tyrosine kinase: it contains tyrosine residues that become phosphorylated upon activation, thereby inducing conformational changes that allow binding of the substrates in the catalytic site of the JAK (Rane and Reddy, 2000). The N-terminal part of the JAKs, containing JH3-7, is involved in association with the receptor. Specifically, JAKs associate with the proline-rich, membrane-proximal box1 and box2 domains of class I cytokine receptors. The JH3-JH4 regions form a structural domain resembling a SH2 domain, but appear not to be implicated in phosphotyrosine-dependent interactions, as SH2 domains typically do. However, this region is structurally important for receptor association and receptor surface expression (Radtke et al., 2005). Finally, the structure of the N-terminal (JH5-JH7) domains resembles that of Four-point-one, Ezrin, Radixin and Moesin (FERM) domains, which are known to mediate protein-protein interactions with for example phosphatidylinositolbisphosphate (PIP2) and inositoltrisphosphate (IP3) (Girault et al., 1998). This region mediates receptor binding and is involved in the maintenance of catalytical activity (Hilkens et al., 2001; Zhou et al., 2001).

![Figure 4: Schematic representation of the JAK structure](image)

**Figure 4: Schematic representation of the JAK structure**

JAKs share seven regions of high similarity (JH1-7). N-terminal domains mediate receptor association; JH1 and JH2 encode respectively a kinase and pseudokinase domain. The ruler underneath indicates the number of AA.
Biological functions – lessons learned from JAK knock-outs

JAKs have non-redundant functions in vivo as is reflected by the phenotypes of JAK knock-out mice (table 3).

**JAK1** - JAK1 knock-out mice die perinatally of a poorly characterized defect that may be neurologic. JAK1-deficient cells are unresponsive to IFNs, γc-dependent cytokines, and to most cytokines signalling through the gp130 receptor subunit (Rodig et al., 1998).

**JAK2** – Targeted disruption of the JAK2 gene results in embryonic lethality due to failure of erythropoiesis. Cells from these mice showed that this kinase is essential for different cytokine responses including IL-3, IL-5, GM-CSF and IFNγ (Neubauer et al., 1998; Parganas et al., 1998). Humans with JAK2 mutations exhibit myeloproliferative disorders (Baxter et al., 2005; James et al., 2005).

**JAK3** - Deletion of the murine JAK3 gene results in a viable phenotype. Nevertheless, these mice suffer from severe combined immune deficiency (SCID) and profound B and T cell defects due to impaired signalling via the γc receptors (Nosaka et al., 1995; Park et al., 1995). Humans that are deficient for JAK3 have SCID and suffer from severe T cell defects, but their B cell populations remain unaffected (Thomis and Berg, 1997).

**TYK2** – In comparison to the other JAKs, TYK2 deficiency in mice caused only a modest phenotype characterized by a shift toward Th2 immune responses and an impaired response to lipopolysaccharide (LPS) (Karaghiosoff et al., 2003). Surprisingly, only subtle defects were observed in IL-12, type I and II IFNs (Karaghiosoff et al., 2000; Karaghiosoff et al., 2003; Shimoda et al., 2000). The role of TYK2 in murine IFN-dependent signalling appears to be limited to specific antiviral activities at low IFN concentrations. By comparison, TYK2-deficient humans exhibit a more severe phenotype. In those individuals, the combined aberrations in IFN, IL-6, IL-10, IL-12 and IL-23 responses are associated with
enhanced allergic and impaired antimicrobial responses (Watford and O'Shea, 2006).

### Table 3: Overview of the major phenotypical differences associated with JAK deficient mice

<table>
<thead>
<tr>
<th>knock-out</th>
<th>major phenotypical differences</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAK1</td>
<td>early postnatal lethality, neurological deficiencies, defective responses to IFNs and cytokines using γc or gp130 receptor subunits</td>
<td>Rodig et al., 1998</td>
</tr>
<tr>
<td>JAK2</td>
<td>embryonic lethality, impaired erythropoiesis</td>
<td>Neubauer et al., 1998; Parganas et al., 1998</td>
</tr>
<tr>
<td>JAK3</td>
<td>viable, fertile, defective lymphoid development</td>
<td>Nosaka et al., 1995; Park et al., 1995</td>
</tr>
<tr>
<td>TYK2</td>
<td>viable, fertile, defective type I and II IFN responses, reduced antiviral response</td>
<td>Karaghiosoff et al., 2000; Shimoda et al., 2000</td>
</tr>
</tbody>
</table>

#### B. The STAT family

The family of mammalian STAT proteins consists of seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. They function as transcription factors which reside predominantly in the cytoplasm in unstimulated cells, most probably as preformed dimers (Mertens et al., 2006; Neculai et al., 2005; Schroder et al., 2004). STAT1, STAT3 and STAT4 can form both homo- and heterodimers while for STAT5 and STAT6 only homodimers have been observed. STAT2 is only functional when complexed with STAT1 or STAT4. Receptor binding and subsequent phosphorylation on tyrosine and serine residues will activate STATs followed by their nuclear translocation. In the nucleus, they initiate transcription by associating with specific response elements in the promoter of target genes. These consensus binding sites are typically palindromic sequences.
The mechanisms underlying the transport of the STATs between the cytoplasm and the nucleus are only partially understood. The predominantly cytosolic localisation for inactive STATs has been shown to reflect a steady-state, where continuous basal nuclear import is balanced by continuous basal nuclear export. After activation, the balance is shifted toward nuclear accumulation and during signal decay toward nuclear export. Translocation of STAT dimers to the nucleus is mediated by specific nuclear localisation signals (NLS) and involves an active nuclear import mechanism depending on Ran and importin-α5 (Fagerlund et al., 2002). An alternative way of nuclear import by which activated receptors mediate nuclear transport of whole receptor/STAT complexes has been proposed (Larkin et al., 2000; Subramaniam et al., 2000). A nuclear export signal (NES) drives the nuclear export of STATs mediated by Ran and exportin 1. STAT dephosphorylation and dissociation from the DNA unmasks the NES sequence and activates the export mechanism (Bhattacharya and Schindler, 2003; McBride and Reich, 2003; Vinkemeier, 2004).

**Structure of STATs**

STATs are about 800 AA long and six structurally and functionally conserved domains have been identified (figure 5). The N-terminus contains a dimer-dimer interaction domain that apparently allows STAT dimers to form tetramers that can cooperatively associate with multiple tandem STAT response elements in the promoter (John et al., 1999; Murphy et al., 2000; Vinkemeier et al., 1996; Xu et al., 1996). It was also reported that this domain is involved in nuclear translocation and STAT deactivation (Strehlow and Schindler, 1998). The N-terminal region is linked to the DNA binding domain by a coiled-coil domain which consists of four helices. This domain can interact with other transcription factors and is also implicated in receptor binding, tyrosine phosphorylation and nuclear export (Begitt et al., 2000; Zhang et al., 2000). The centrally located DNA binding domain harbours a typical β-barrel with an Ig fold, a structure found in various transcription factors, like NF-κB and p53. Obviously, it is implicated in DNA association but there are only little direct interaction sites (Becker et al., 1998; Chen et al., 1998).
Figure 5: Schematic representation of the STAT structure
P indicates phosphorylated residues. The ruler underneath indicates the number of AA.

The SH2 domain is crucial for docking of the STAT to the phosphorylated tyrosine motifs in the receptor or in JAK kinases and it also mediates dimerisation of the STATs. The SH2 domain consists of a β-sheet flanked by two α-helices, which form a pocket structure. A conserved arginine residing in this pocket is essential for the interaction with phosphotyrosine residues (Chen et al., 1998). C-terminal to the SH2 domain, STATs have a conserved tyrosine residue which becomes phosphorylated by the JAK kinases upon receptor binding. Dimerization of the activated STATs is based on the reciprocal interaction of the SH2 domain with this phosphorylated tyrosine. The more variable C-terminus of STATs encodes a transcription activation domain (TAD). This divergence provides an opportunity to associate with distinct transcriptional regulators. This domain can be serine phosphorylated, which enhances the transcription of some genes (Decker and Kovarik, 2000).

Biological functions – lessons learned from STAT knock-outs

Specific deletion of STAT genes revealed distinctive functions for the various STAT proteins. An overview of the different knock-out mice phenotypes is given in table 4.

STAT1 - STAT1 deficient mice confirmed the pivotal role that STAT1 plays in the biological response to both type I and type II IFNs. These mice are highly
susceptible to bacterial and viral infections (Durbin et al., 1996; Meraz et al., 1996). Consistent with this, naturally occurring mutations in human STAT1 exhibit increased susceptibility to viral and bacterial infections (Chapgier et al., 2006). STAT1 seems to be also implicated in non immune-responses, like chondrocyte proliferation (Sahni et al., 1999) and IFNγ-mediated growth inhibition, as was revealed by the enhanced tumor susceptibility of STAT1<sup>-/-</sup> mice (Shankaran et al., 2001).

Table 4: Overview of the major phenotypical differences associated with STAT deficient mice

<table>
<thead>
<tr>
<th>knock-out</th>
<th>major phenotypical differences</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT1</td>
<td>viable, IFN responses absent, highly sensitive to viral/microbial infection</td>
<td>Durbin et al., 1996, Meraz et al., 1996</td>
</tr>
<tr>
<td>STAT2</td>
<td>viable, type I IFN responses impaired, defective antiviral response</td>
<td>Park et al., 2000</td>
</tr>
<tr>
<td>STAT3</td>
<td>embryonic lethality</td>
<td>Takeda et al., 1997</td>
</tr>
<tr>
<td>STAT4</td>
<td>viable, defective Th1 differentiation</td>
<td>Kaplan et al., 1996b, Thierfelder et al., 1996</td>
</tr>
<tr>
<td>STAT5a</td>
<td>viable, impaired mammalian gland development</td>
<td>Liu et al., 1997</td>
</tr>
<tr>
<td>STAT5b</td>
<td>viable, impaired growth</td>
<td>Udy et al., 1997</td>
</tr>
<tr>
<td>STAT6</td>
<td>viable, defective Th2 differentiation, impaired B cell functions</td>
<td>Kaplan et al., 1996a, Takeda et al., 1996</td>
</tr>
</tbody>
</table>

**STAT2** - STAT2 null mice also display deficiencies in antiviral responses. Their defective phenotype is principally due to impaired type I IFN signalling that is dependent on STAT1/STAT2 heterodimers (Park et al., 2000). STAT1/STAT2 double knock-out mice are totally refractory to both classes of IFNs and are more vulnerable to infections than either single knock-out mice.

**STAT3** - STAT3 gene targeting has underscored the vital developmental role for STAT3 as deficient mice die embryologically before gastrulation (Takeda et al., 1996).
In contrast, tissue-specific knock-outs have highlighted an important anti-inflammatory role for STAT3. Macrophages and neutrophils depleted of STAT3 exhibit a higher susceptibility to endotoxin shock and a higher production of inflammatory cytokines due to impaired IL-10 responsiveness (Takeda et al., 1999). STAT3 deficiency in T cells abrogates their proliferative response to IL-6 (Takeda et al., 1998). STAT3 deficient hepatocytes have an impaired induction of acute phase genes in response to IL-6 (Alonzi et al., 2001). Finally, lack of STAT3 in keratinocytes results in impaired wound healing and hair growth (Sano et al., 1999). Consistent with these findings, STAT3 directs the expression of pro-proliferative and anti-inflammatory cytokines, thereby contrasting with the anti-proliferative and pro-inflammatory activities of STAT1.

**STAT4** - Genetic targeting of STAT4 has revealed a crucial role in directing the biological response to IL-12 and in regulating the differentiation of Th1 and Th2 cells. STAT4 deficient mice have defects in IL-12 mediated responses by natural killer cells and T lymphocytes, including the production of IFNγ, mitogenesis, enhancement of natural killer cytolytic function and Th1 differentiation (Kaplan et al., 1996b; Thierfelder et al., 1996). More recently, STAT4 was shown to be important in the IL-23 dependent expansion of Th17 cells and associated autoimmunity (Hunter, 2005).

**STAT5** - Although extensive sequence similarity between STAT5a and STAT5b (~96% AA identity) explains their functional redundancy observed in vitro, the phenotypes of the single knock-outs reflect striking differences. STAT5a null mice are predominantly defective in PRL-mediated gland development (Liu et al., 1997), whereas STAT5b deficiency causes aberrations in sexual dimorphic growth, reminiscent of the phenotype of GH deficient mice (Udy et al., 1997). STAT5a/b double knock-outs exhibit a more severe phenotype. Many of these mice die within weeks after birth. The surviving mice are smaller and infertile with mammary gland defects, underscoring the essential role of the STAT5 proteins in GH and PRL responses (Teglund, 1998 #34). Recent STAT5a/b gene targeting studies have revealed an important role for the STAT5 molecules in erythropoiesis and lymphopoiesis (Yao et al., 2006).
**STAT6** - STAT6 deficient mice have confirmed a critical role for STAT6 in the IL-4 and IL-13 dependent polarization of naive lymphocytes into Th2 effector cells, as well as in mast cell activation. The gene targeting studies have also highlighted an important role for STAT6 in promoting B cell function, including proliferation, maturation, and MHC-II and IgE expression (Kaplan et al., 1996a; Takeda et al., 1996).

**Table 5: Cytokine-specific JAK and STAT activation**
Based on the composition of receptor chains and their use of JAKs and STATs, cytokine receptors can be divided into five subfamilies. Adapted from (O'Sullivan et al., 2007).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>JAK Kinase</th>
<th>STATs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNα/β</td>
<td>Tyk2, JAK1</td>
<td>STAT1, STAT2 (STAT3, STAT5)</td>
</tr>
<tr>
<td>IFNγ</td>
<td>JAK1, JAK2</td>
<td>STAT1 (STAT3, STAT5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>Tyk2, JAK1</td>
<td>STAT3 (STAT1, STAT5)</td>
</tr>
<tr>
<td>gp130 family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3 (STAT5)</td>
</tr>
<tr>
<td>IL-11</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3 (STAT5)</td>
</tr>
<tr>
<td>IL-12</td>
<td>JAK2, TYK2</td>
<td>STAT4</td>
</tr>
<tr>
<td>OSM</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3 (STAT5)</td>
</tr>
<tr>
<td>LIF</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3 (STAT5)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT3 (STAT1, STAT5)</td>
</tr>
<tr>
<td>Leptin</td>
<td>JAK1, JAK2</td>
<td>STAT3 (STAT1, STAT5)</td>
</tr>
<tr>
<td>γc family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>JAK1, JAK3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-4</td>
<td>JAK1, JAK3</td>
<td>STAT6</td>
</tr>
<tr>
<td>IL-7</td>
<td>JAK1, JAK3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-9</td>
<td>JAK1, JAK3</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-15</td>
<td>JAK1, JAK3</td>
<td>STAT5</td>
</tr>
<tr>
<td>βc family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>IL-5</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>Single chain family</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epo</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>GH</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>PRL</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
<tr>
<td>TPO</td>
<td>JAK2</td>
<td>STAT5</td>
</tr>
</tbody>
</table>

Chapter 1: Cytokine receptors and signal transduction
Almost 40 cytokine receptors signal through combinations of four JAK and seven STAT family members, suggesting commonality in the JAK-STAT signalling cascade. Based on their use of JAKs and STATs, cytokine receptors are divided into five subfamilies (overview in table 4). Although only a limited number of JAK and STAT proteins are implicated, activation of the pathway by divergent stimuli will lead to unique biological responses.

The specificity of JAK-STAT signalling first originates from the preferential association of one JAK (or JAK combination) to certain receptor classes. Second, each receptor will use its own set of STAT molecules, leading to transcription of a defined collection of target genes. STAT specificity is largely determined by the binding preference of their SH2 domains for phosphorylated tyrosine residues on specific receptors. Third, posttranslational modifications and formation of STAT heterodimers, tetramers or higher order complexes expands the range of STAT/DNA binding opportunities. Fourth, specific gene expression is refined by genomic accessibility and other cofactors that act in synergy with STATs. These cofactors are often cell type specific or are activated by other receptor signalling pathways.

Despite extensive study, there remain substantial gaps in understanding how the JAK-STAT cascades are activated and regulated. There is for example still a lack of structural information on the cytoplasmatic domains of cytokine receptors, particularly in association with JAKs and STATs. We also do not fully understand how specificity in gene expression is generated by receptors that use identical JAK and STAT members. Further, it is intriguing that despite the large amount of cytokine receptors, the number of JAKs or JAK combinations remained so small during evolution.

IV. A selection of other cytokine-induced signalling pathways

In mammals, the JAK-STAT pathway is the principal signalling mechanism for a wide array of cytokines and growth factors. However, other pathways are also contributing to the signal transduction originating from cytokine receptors. A number of adaptor molecules can be recruited to the receptor or the kinase and may provide a link to other signalling pathways upon JAK-dependent phosphorylation. This
further emphasizes the major role of JAK kinases in the total signalling event induced by a cytokine. Most cellular responses are derived from a combined effort of several activated signalling pathways, which are often intertwined by cross-talk actions. An example is the potential role of mitogen-activated protein kinases (MAPK) in activation of STAT proteins by serine phosphorylation.

**Figure 6: Schematic overview of the MAPK and PI3K/Akt pathways.**
Transcription factors that are activated in the different pathways are shown in yellow.

**The MAPK pathway** - The MAPK signalling cascade is initiated by receptor and/or JAK binding of a set of adaptors including SH2 domain containing phosphatase-2 (SHP2), growth receptor bound protein 2 (Grb2), insulin receptor substrate (IRS) and Shc to the activated receptor complex. As illustrated in figure 6, binding of Grb2 can link the receptor to a guanine nucleotide exchange factor like son of sevenless (SOS). The latter protein activates membrane-anchored small GTP binding proteins
like Ras, which in turn stimulate the core unit of this cascade composed of series of serine-threonine kinases: MAPKKK (Raf), MAPKK (MEK1/2) and MAPK (Erk1/2) (Morrison and Cutler, 1997; Rubinfeld and Seger, 2005). Three major groups of MAP kinases exist: the p38 MAP kinase family, the extracellular signal-regulated kinase (Erk) family, and the c-Jun NH2-terminal kinase (JNK) family (Chang and Karin, 2001; Johnson and Lapadat, 2002). Activation of the different MAPKs will generate diverse, often conflicting cellular responses including inflammation, cell growth, differentiation and survival (Dong et al., 2002; Platanias, 2003).

The PI3K/Akt pathway - Another example is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that induces cell growth and anti-apoptotic mechanisms upon stimulation by many cytokines. The PI3K family is a group of related lipid kinases, of which the classical form is built up of two subunits, a regulatory (p85) and a catalytic subunit (p110) (Fruman et al., 1998). The p85 subunit can interact with phosphotyrosines of activated receptors or adaptor proteins and thereby recruits the p110 subunit to the membrane, where it phosphorylates phosphoinositides (Fruman et al., 1998; Okkenhaug and Vanhaesebroeck, 2001). These phosphorylated lipids serve as docking sites for several signalling molecules containing a pleckstrin-homology (PH) domain, like the Ser-Thr kinases Akt and phosphoinositide-dependent kinase 1 (PDK1) (Coffer et al., 1998). Phosphorylation of Akt by PDK1 and other kinases stimulates the catalytic activity of Akt (Anderson et al., 1998; Brazil et al., 2002), resulting in the phosphorylation of other signalling proteins, thereby altering their function in cell growth and survival (Krasilnikov, 2000). This pathway is often deregulated in cancer, since aberrations in this signalling can lead to uncontrolled cell growth (Fresno Vara et al., 2004; Hennessy et al., 2005; Krasilnikov, 2000).

Other pathways - Activated JAK kinases can phosphorylate other adaptor proteins including Vav, resulting in the activation of the Rho family of GTPases and phospholipase C gamma (PLCγ), which will induce the release of Ca²⁺ from intracellular stores and activate the family of phosphokinase C (PKC) molecules (Carpenter and Ji, 1999; Nobes and Hall, 1995; Patterson et al., 2005).
V. References


Chapter 1: Cytokine receptors and signal transduction
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Cytokines are involved in many vital biological processes. Therefore, it is of crucial importance that cytokine signals are rapidly and finely tuned to avoid physiological derangements. In order to keep signalling events under tight control, cellular mechanisms have evolved to ensure that adequate signalling thresholds are achieved and maintained for the correct duration. Regulation of the initiation, duration and magnitude of cytokine signalling occurs at multiple levels: limiting the availability of cytokine to initiate a response, regulating the expression and half-life of cell surface receptor components, controlling the intracellular signal transduction machinery and transcriptional control. Some of the most important mechanisms and protein families involved in negative regulation of signalling will be discussed in this chapter (figure 7). Since the scope of the thesis concerns suppressor of cytokine signalling (SOCS) proteins, the following chapter will discuss this important family of regulatory proteins in greater detail.

I. Soluble receptors

Soluble receptors regulate biological responses by functioning as agonists or antagonists of cytokine signalling. A soluble counterpart of a cytokine receptor can be generated in two distinct ways: alternative mRNA splicing or proteolytic cleavage, which sheds the ectodomain of membrane-spanning receptors. Secreted receptor subunits were initially proposed to be antagonists, blocking or reducing cytokine potency by competing with their membrane-anchored homologues for common ligands. In this respect, the activity of IL-18 can be neutralized by the IL-18-binding protein (IL-18BP) that binds IL-18 with high affinity (Novick et al., 1999). In many cases however, soluble receptors are protecting the ligand from degradation or excretion. In this case, the receptor variant acts as a carrier for the ligand, thereby increasing the half-life of the cytokine. This function is for example of particular interest in case of the GH, where the site of cytokine production is remote from the
target tissue (Baumann, 1995). A secreted receptor may also substitute for an absent endogenous binding subunit, thereby converting a ligand-resistant cell into a sensitive one. This principle of transsignalling was originally described for IL-6 signalling. This cytokine can bind its soluble receptor and signal through the gp130 receptor subunit on a target cell, not expressing the membrane bound IL-6R subunit (Heinrich et al., 1995; Peters et al., 1996).

II. Internalisation and degradation

Internalisation and subsequent degradation of the receptor complex is an effective mechanism to turn off cytokine actions. Several internalisation mechanisms using the clathrin-coated pit pathway or clathrin-independent ways have been described. Frequently, a di-leucine motif in the cytoplasmatic part is found to be involved in receptor internalization (Aarts et al., 2004; Dittrich et al., 1994; Thiel et al., 1999). Internalised receptors are routed to the lysosomes and degraded, although receptor subunits may be recycled back to the plasma membrane like was for example demonstrated for the IL-2Rα (Hemar et al., 1995).

The ubiquitin/proteasome system is another way of dismantling the receptor complex. Destruction of a protein via this system involves two successive steps: attachment of a chain of ubiquitin molecules to the target, resulting in recognition and degradation of the poly-ubiquinated protein by the 26S proteasome complex with release of reusable ubiquitin. Ubiquitination is described in more detail in text box2. Several signalling molecules are susceptible to ubiquitination, predestining them for proteasomal degradation (Callus and Mathey-Prevot, 1998; Kim and Maniatis, 1996; Wang et al., 2000; Yu and Burakoff, 1997). In this context, it was reported that proteasome-dependent degradation of receptor complexes including the GHR and EpoR can be mediated by members of the family of SOCS proteins (see further) (Verdier et al., 1998). Beside proteasomal degradation, SOCS proteins can also direct the internalisation and routing of cytokine receptors as was for example demonstrated for the GHR, granulocyt-colony stimulating factor receptor (G-CSFR) and epidermal growth factor receptor (EGFR) (Irandoust et al., 2007; Kario et al., 2005; Landsman and Waxman, 2005).
Text box 2: The Ubiquitination System

Ubiquitin (Ub) is a highly conserved protein of 76 amino acids that functions as a protein-modifier by covalent coupling via its C-terminus to target proteins. This ubiquitination marks these proteins, thereby affecting their stability, activity and/or subcellular localization. The binding of Ub to its substrates is a multi-step process that involves successive action of three classes of enzymes. The Ub-activating enzyme E1 activates Ub by forming a thioester bond. Activated Ub is then transferred to an Ub-conjugating enzyme E2 by transthiolation. Finally, an Ub ligase E3 couples Ub to a lysine residue or the N-terminus of the E3 associated target protein (Breitschopf et al., 1998; Glickman and Ciechanover, 2002). The association of the E3 ligases with their target protein determines substrate specificity for ubiquitination. Ubiquitination is a reversible and dynamic process, balanced by deubiquitinating enzymes (DUBs) that decouple Ub (Weissman, 2001).

Proteins can be modified by a single or multiple Ub moieties, termed mono-, multi- or poly-ubiquitination. Poly-Ub chains are formed by coupling of Ub to any of the seven lysines in the previously conjugated Ub. The different types of ubiquitination are proposed to direct the target protein for different cellular events. Mono-ubiquitination is for instance involved in receptor internalisation and histone regulation (Belouzard and Rouille, 2006; Hicke and Dunn, 2003; Osley, 2004). In many cases, poly-ubiquitination results in protein turnover by the proteasome, but it can also be involved in ribosomal function, DNA repair or signal transduction (Geetha et al., 2005; Spence et al., 2000; Wilkinson et al., 2005). Lys48-linked poly-Ub chains usually target proteins for destruction by the 26S proteasome (Kim and Rao, 2006; Pickart, 2001; Thrower et al., 2000). A well-studied example is the degradation of the hypoxia-inducible transcription factor (HIF)-1α by the Von Hippel Lindau (VHL) tumor suppressor. The turnover of HIF-1α is oxygen-regulated: under normal oxygen conditions, hydroxylation of HIF-1α will result in its recognition and destruction by VHL (Hon et al., 2002; Iliopoulos et al., 1996; Iwai et al., 1999; Lisztwan et al., 1999; Salceda and Caro, 1997). Conversely, in hypoxic conditions, HIF-1α is refractory to ubiquitination by VHL and will be able to induce expression of hypoxia-inducible genes regulating angiogenesis and erythrocytosis (Huang et al., 1996; Semenza, 1999; Wang and Semenza, 1993). VHL acts as the substrate recognition unit for an E3 ubiquitin ligase complex. It contains a substrate-interaction domain and a SOCS box which is conserved amongst different protein families, including the SOCS protein family. The SOCS box is proposed to serve as a common link of these proteins with the proteasomal degradation system (Hilton et al., 1998; Kile et al., 2002; Zhang et al., 1999). A small conserved motif in the SOCS box, named the B/C box, functions as a docking site for the adaptor molecules Elongin C and B (Aso et al., 1996; Duan et al., 1995; Kibel et al., 1995). Together with a Cullin box motif that is situated downstream of the B/C box (Kamura et al., 2004), the Elongin B/C dimer will bridge the substrate to a Cullin2 scaffold protein. Cullin2 in turn binds a RING finger-containing protein Rbx1 and together these proteins form an E3 ubiquitin ligase complex which will ultimately direct the ubiquitination and proteasomal degradation of HIF-1α (Iwai et al., 1999; Pause et al., 1997). Poly-ubiquitination that is not based on Lys48-linked Ub chains can be associated with processes as diverse as protein translation, activation of transcription factors and DNA repair (Geetha et al., 2005; Kim and Rao, 2006; Spence et al., 2000; Wilkinson et al., 2005).

Besides Ub, several other Ub-like (Ubl) proteins can also modify target proteins using a similar mechanism for their covalent conjugation. Examples include SUMO (small Ub-related modifier), ISG15 (interferon stimulated gene product 15), Nedd8 and Atg8, and these Ubls function as critical regulators of many cellular processes such as transcription, DNA repair, signal transduction and cell-cycle control (Dohmen, 2004; Kerscher et al., 2006; Ritchie and Zhang, 2004). Ub-like domains with a characteristic Ub fold can occur as stable regions within other proteins, as was demonstrated for Elongin B (Stebbins et al., 1999). These domains do not couple to other proteins but probably function in Ub-mediated processes (Pickart and Eddins, 2004; Weissman, 2001).
The turnover of some receptors such as the EGFR and IFNaR1 is dependent on both lysosomal and proteasomal activity (Kumar et al., 2003; Longva et al., 2002). Also for the EpoR it was suggested that the receptor complex is degraded by two proteolytic systems that proceed successively: the proteasome removes part of the intracellular domain of the EpoR at the cell surface, and the remaining part of the receptor-hormone complex is degraded in the lysosomes (Walrafen et al., 2005).

A number of receptors are subjected to regulated intramembrane proteolysis (RIP). After ectodomain cleavage, the cytoplasmatic part is recognized by intracellular proteases, resulting in proteolysis and release of the intracellular domain. This domain may then translocate to the nucleus, where it participates in transcriptional activation or functions as a substrate for proteasomal degradation.

### III. Phosphatases

Since phosphorylation is a crucial trigger in the activation of cytokine signalling cascades, dephosphorylation by protein tyrosine phosphatases (PTPs) is an obvious mechanism that contributes to negative control.

**SHP-1 and -2** - SH2 domain containing phosphatase (SHP)-1 and -2 are constitutively expressed phosphatases responsible for the dephosphorylation and inactivation of JAK kinases. Both proteins bind to either phosphorylated JAKs or receptors via two src homology (SH2) domains. Whereas SHP-2 is broadly expressed, SHP-1 expression is restricted to haematopoietic cells (Ahmad et al., 1993; Yi et al., 1992). The important role of the SHP-1 and -2 phosphatases is underscored by the lethal phenotype of their knock-outs. Mice with a naturally occurring mutation in the SHP-1 gene die shortly after birth, due to multiple abnormalities in immune responses (Kamata et al., 2003; Shultz et al., 1997). SHP-2 deficient mice are embryonic lethal and this is caused by defects in EGF signalling (Qu et al., 1999; Saxton et al., 1997). A positive regulatory role for SHP-2 in the MAPK pathway was reported for several cytokine systems: SHP-2 becomes phosphorylated itself, which in turn can lead to docking of the Grb2/Sos complex, thereby activating the MAPK pathway (Bennett et al., 1994; Cunnick et al., 2002).
SHP-2 was also reported to interact with STAT molecules and positively regulate their phosphorylation and activity (Chughtai et al., 2002). In contrast, SHP-2 may be responsible for tyrosine dephosphorylation of STAT5 in the cytosolic compartment (Yu et al., 2000).

Figure 7: Schematic overview of the negative regulation of cytokine signalling.
For detailed information, see text.
**Others** - Genetic and biochemical approaches have implicated several other phosphatases in the inhibition of JAK kinase activity, including CD45 (Irie-Sasaki et al., 2001), PTPεC (Tanuma et al., 2000) and PTP1B (Elchebly et al., 1999; Myers et al., 2001). Similar approaches have underscored a role for SHP-2, PTP1B, TC45 and PTP-basophile like (PTP-BL) in STAT dephosphorylation (Aoki and Matsuda, 2000; Kerscher et al., 2006; Lund et al., 2005; Mustelin et al., 2005; Nakahira et al., 2007; ten Hoeve et al., 2002), which appears to be critical for STAT nuclear export (McBride and Reich, 2003; Vinkemeier, 2004).

**IV. PIAS proteins**

The Protein inhibitors of activated STAT (PIAS) protein family consists of four members; PIAS1, PIAS2 (also referred to as PIASx), PIAS3 and PIAS4 (also referred to as PIASy) (Shuai and Liu, 2005). Except for PIAS1, two isoforms were identified for each PIAS protein. These proteins have a central Zn-binding RING-finger domain, a conserved SAP (SAF-A/Acinus/PIAS) domain at the N-terminus, and a less conserved C-terminal part. The latter domains are involved in substrate binding. PIAS proteins bind to activated STAT dimers and prevent them from binding DNA. STAT1 and STAT3 are specifically inhibited by respectively PIAS1 and PIAS3 (Chung et al., 1997; Liu et al., 1998). Yet, PIAS2 and PIAS4 recruit additional co-repressing factors to inhibit the STAT transcriptional activity of respectively STAT4 and STAT1 (Arora et al., 2003; Liu et al., 2001). Furthermore, PIAS proteins have been shown to function as E3 type SUMO ligases that conjugate SUMO moieties to target proteins (Jackson, 2001). Although there is evidence that STATs can be modified by sumoylation (Rogers et al., 2003; Schmidt and Muller, 2003), the function of that modification in negative regulation is not yet clear.
V. References


SOCS are a family of intracellular proteins that play a major role in the regulation of cytokine responses. The founding member of the SOCS family, the cytokine-inducible SH2 domain containing (CIS) protein, was cloned in 1995 and was originally identified as an immediate early response gene induced in haematopoietic cells in response to Epo or IL-3 (Yoshimura et al., 1995). Following that report, SOCS1 was identified by three different groups in 1997 as a novel JAK regulatory protein (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Database searches led to the identification and cloning of 6 additional SOCS proteins based on sequence homology (Hilton et al., 1998; Masuhara et al., 1997; Starr et al., 1997). So at present, the SOCS family counts eight members: CIS and SOCS1 through SOCS7.

I. Expression of SOCS proteins

SOCS proteins are often expressed at low or undetectable levels in resting cells. They are rapidly up-regulated in response to a broad spectrum of cytokines and, in turn, control the duration and intensity of cytokine responses by blocking various aspects of the signalling pathways. SOCS proteins function in a typical negative feedback loop, since they can down-modulate the signalling pathway that stimulates their production. The pattern of SOCS expression by a particular cytokine tends to vary according to the cell type or tissue. The induced SOCS proteins can attenuate responses of various cytokines and may be involved in inhibitory cross-talk between different cytokine systems, providing a mechanism by which concurrent signalling processes can modulate each other. An overview of SOCS induction patterns and inhibition of cytokine signalling is given in table 6. Although SOCS proteins are induced by a range of cytokines and show a high structural and functional overlap, large evidence indicates the potential of specific SOCS proteins to modulate cytokine signalling with exquisite control. The SOCS expression is tightly regulated
through multiple mechanisms in order to avoid inappropriate interference with physiological responses (reviewed in chapter 11).

**Table 6: SOCS induction, inhibition patterns and cross-regulation**

<table>
<thead>
<tr>
<th><strong>SOCS</strong></th>
<th><strong>Induced by</strong></th>
<th><strong>Inhibits signal transduction of</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CIS</td>
<td>Leptin, Epo, GH, PRL, LIF, G-CSF, GM-CSF, TPO, CNTF, IFNα, IFNγ, IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-12, IL-13</td>
<td>Leptin, Epo, GH, PRL, IGF1, G-CSF, IL-2, IL-3</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Leptin, Epo, GH, PRL, LIF, insulin, G-CSF, GM-CSF, CNTF, IFNαβ, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-13, LPS, CpG DNA</td>
<td>Leptin, Epo, GH, LIF, PRL, Epo, Insulin, TNFα, OSM, TPO, IGF1, IFNαβ, IFNγ, IL-2, IL-4, IL-6, IL-7, IL-12, IL-15</td>
</tr>
<tr>
<td>SOCS2</td>
<td>Leptin, GH, PRL, Insulin, Estrogen, EPO, CNTF, G-CSF, GM-CSF, TNFα, LIF, IFNα, IFNγ, IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, Lipoxins</td>
<td>Leptin, GH, PRL, LIF, IFNγ, IGF1, EGF, IL-6</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Leptin, Epo, GH, PRL, Insulin, GM-CSF, M-CSF, G-CSF, CNTF, TPO, TNFα, LIF, IFNα, IFNγ, IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, LPS, CpG DNA</td>
<td>Leptin, Epo, GH, PRL, OSM, CNTF, IGF1, Insulin, LIF, IFNαβ, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-9, IL-11</td>
</tr>
<tr>
<td>SOCS4</td>
<td>EGF</td>
<td>EGF</td>
</tr>
<tr>
<td>SOCS5</td>
<td>EGF, IL-6</td>
<td>EGF, LIF, IL-4, IL-6</td>
</tr>
<tr>
<td>SOCS6</td>
<td>Insulin, Kit</td>
<td>Leptin, Insulin, Kit</td>
</tr>
<tr>
<td>SOCS7</td>
<td>GH, PRL, Insulin, IL-6, IFNγ, IL-1β</td>
<td>Leptin, GH, PRL, Insulin</td>
</tr>
</tbody>
</table>

SOCS can be induced by stimuli other than cytokines, including growth factors, chemokines, hormones, pathogens and their products such as CpG DNA or LPS (Baetz et al., 2004; Crespo et al., 2000; Dalpke et al., 2001; Dogusan et al., 2000; Krebs and Hilton, 2003; Leong et al., 2004; Stoiber et al., 1999). SOCS expression was also found to be developmentally regulated in the absence of cytokine signalling (Illogumaran et al., 2004; Kubo et al., 2003).

STAT proteins are the major regulators of SOCS gene expression. STAT binding sequences were identified in the SOCS promoter region and electrophoretic mobility shift assays confirmed STAT association to these motifs (table 7). In some cases, STAT induced SOCS expression is indirect. STAT1 is for example indirectly implicated in IFNγ mediated upregulation of SOCS1 since it drives expression of the

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Chapter 3: The SOCS protein family

- 46 -
interferon regulatory factor-1 (IRF-1) transcription factor which is responsible for SOCS1 induction (Saito et al., 2000).

Table 7: STAT-responsive elements in SOCS promoters

<table>
<thead>
<tr>
<th>STAT binding elements</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CIS</strong></td>
<td>STAT5</td>
</tr>
<tr>
<td><strong>SOCS1</strong></td>
<td>STAT1, STAT3, STAT6</td>
</tr>
<tr>
<td><strong>SOCS2</strong></td>
<td>STAT5</td>
</tr>
<tr>
<td><strong>SOCS3</strong></td>
<td>STAT1, STAT3, STAT5</td>
</tr>
</tbody>
</table>

(SOCS proteins not only control kinetics and magnitude of signalling but can also be involved in the shaping of the cytokine responses. For example, SOCS3 regulates both the quantity and type of STAT signalling generated from the pro-inflammatory IL-6R. Loss of SOCS3 will alter the functional outcome of IL-6 signalling by prolonging STAT activation. IL-6 then behaves more like the immunosuppressive cytokine IL-10 and induces IFN gene expression owing to respectively an excess of STAT3 and STAT1 phosphorylation (see below) (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003).

**II. SOCS protein structure and molecular mechanisms of action**

Comparative sequence analysis of the SOCS members revealed that CIS and SOCS2, SOCS1 and -3, SOCS4 and -5 and finally SOCS6 and -7 form closely related pairs (Hilton et al., 1998). CIS and SOCS2 are the most related with around 35% of AA identity while the rest of SOCS pairs share approximately 25% of AA sequences. SOCS homologues were also identified in *D. melanogaster* and *C. elegans* (Kile et al., 2002; Starr et al., 1997). The general structure of SOCS proteins is evolutionary well preserved and common features include an N-terminal
pre-SH2 domain, a central SH2 domain and a C-terminal SOCS box (figure 8). The N-terminal part varies in length and sequence similarity while the SH2 domain and SOCS box are most conserved.

**Figure 8: Domain structure of the SOCS protein family**
The major structural characteristic of the SOCS family is the presence of two domains with relatively well conserved AA sequence: an SH2 domain in the middle portion and a SOCS box at the C-terminus. Only SOCS1 and -3 possess a KIR immediately upstream of the SH2 domain. Conserved tyrosines in the SOCS box are represented by a black line.

*The N-terminal domain: JAK kinase inhibition.*

The N-terminus varies greatly in length among the SOCS members and contains no homology to known structural domains. Nevertheless, an extended SH2 subdomain (ESS) helix was identified as a conserved structural element in CIS and SOCS1-3 and appeared to be critical for high affinity SH2 substrate interactions (Yasukawa et al., 1999). Both SOCS1 and 3 can be distinguished from the other SOCS proteins by an additional small kinase inhibitory region (KIR) of 12 AA located in the N-terminal portion and involved in inhibition of the JAK kinases. This region was found functionally interchangeable between the two SOCS suggesting a common inhibitory mechanism (Nicholson et al., 1999). The KIR region displays some sequence similarity with the activation loop of JAK2, suggesting that it acts as a pseudosubstrate by mimicking the activation loop that regulates access to the
catalytic groove (Yasukawa et al., 1999). Based on a structural model of SOCS1 in complex with JAK2 it was proposed that the KIR region suppresses JAK activity by obstructing the access of both ATP and substrate to their respective binding sites (Giordanetto and Kroemer, 2003). Although the SH2 domain of SOCS3 does not have a high affinity for JAK kinases, the KIR domain of SOCS3 shows a stronger potential for both binding and inhibition of JAKs than that of SOCS1 (figure 9) (Sasaki et al., 1999). The wide range of actions exhibited by SOCS1 and SOCS3 is most probably due to their additional ability to inhibit the catalytic activity of JAK kinases.

Chapter 3: The SOCS protein family
The SH2 domain: substrate specificity and competition for receptor motifs.

The central SH2 domain determines the target of the SOCS protein. It allows interaction with phosphorylated tyrosine residues of other proteins, like receptors, JAKs or adaptors. This way, SOCS proteins can exert their inhibitory effects by competing with other signalling molecules including STATs for phosphorylated tyrosine motifs in the receptor complex. Because of direct association and steric hindrance by the SOCS molecules, the docking sites then become inaccessible for other signal transducers (figure 9).

SOCS1 directly interacts with all JAK members, thereby inhibiting their catalytic activity (Endo et al., 1997; Naka et al., 1997; Nicholson et al., 1999). It targets the phosphotyrosine at position Y1007, which lies within the activation loop of JAK2 (Giordanetto and Kroemer, 2003; Yasukawa et al., 1999). Activation of the kinase is dependent on phosphorylation of this particular tyrosine. The SOCS1 SH2 domain is sufficient for JAK2 interaction. However, as mentioned above, an additional region of approximately 30 residues immediately N-terminal to the SH2 domain (the ESS) and the KIR are additionally required for high affinity binding and inhibition of JAK2 activity (Giordanetto and Kroemer, 2003; Yasukawa et al., 1999).Remarkably, SOCS1 has also been shown to bind directly to the type I and type II IFN receptors, which might provide a very efficient inhibitory effect of SOCS1 on IFN signalling (Fenner et al., 2006; Qing et al., 2005). SOCS3 showed only weak affinity for JAK2 itself and it is proposed to bind with phosphotyrosine motifs in the receptor close to the kinase to inhibit its activity through the KIR domain (Suzuki et al., 1998). High affinity interaction of SOCS3 was demonstrated for gp130 (Nicholson et al., 2000) and other related receptors (Bjorbaek et al., 2000; Eyckerman et al., 2000; Hortner et al., 2002; Ram and Waxman, 1999; Yamamoto et al., 2003). NMR and crystal structures of SOCS3 with a bound gp130 phosphotyrosine peptide showed the importance of the residue at position Y+3 (Babon et al., 2006; Bergamin et al., 2006). While residues at positions Y+1 and Y+2 are solvent exposed, the hydrophobic character of the pocket residue is conserved in all SOCS proteins, suggesting a similar hydrophobic pocket. An overview of the reported phosphotyrosine binding preferences of the SH2 domains of SOCS1 and SOCS3 is given in table 8. The SH2 domains of SOCS2 and CIS are reported to interact
primarily with phosphotyrosines of receptors such as the EpoR or the GHR, of which several are known to be STAT recruitment sites (Ram and Waxman, 1999; Verdier et al., 1998).

Table 8: Phosphotyrosine binding preferences of SOCS1 and SOCS3

<table>
<thead>
<tr>
<th></th>
<th>-2</th>
<th>-1</th>
<th>Y(p)</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>+5</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS1</td>
<td>E</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>E</td>
<td>(Giordanetto and Kroemer, 2003)</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Y</td>
<td>S/A/V/Y/F</td>
<td>Φ</td>
<td>V/I/L</td>
<td>Φ</td>
<td>H/V/I/Y</td>
<td></td>
<td></td>
<td>(De Souza et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>E</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
<td>V</td>
<td>V</td>
<td>(Babon et al., 2006)</td>
</tr>
</tbody>
</table>

The SOCS box: proteasomal targeting and protein stability

As mentioned before, the C-terminal SOCS box is conserved throughout the SOCS family, thus suggesting an important role for this region in the function and regulation of SOCS proteins. Different SOCS box containing protein families have been identified. Instead of possessing SH2 domains they contain other protein-protein interacting motifs, such as ankyrin repeats, SPRY domains or WD40 repeats (Hilton et al., 1998). Clues to understand the function of the SOCS box came from the initial finding that the Von Hippel-Lindau (VHL) tumor suppressor protein interacts with its Elongin B/C complex via the SOCS box, linking VHL to the ubiquitin/proteasome degradation system (figure 9) (Iwai et al., 1999; Kibel et al., 1995). The Elongin B/C-Cullin-SOCS box (ECS) complex was characterized as a family of E3 ubiquitin ligases in which the SOCS box protein acts as the substrate recognition unit of the complex (Kile et al., 2002). The SOCS box domain of these proteins mediates the interaction with Elongin C by the B/C box, a xxLxxxCxxx(A/I/L/V) conserved sequence (Aso et al., 1996; Duan et al., 1995; Kamura et al., 1998; Kibel et al., 1995). Recently, the B/C box was characterized as a (S,T,P,L)xxx(C,S,A)xxxΦ sequence (Mahrou et al., 2008). Elongin B binds Elongin C and this dimer acts as a linker that bridges the substrate recognized by the SOCS box protein to a Cullin scaffold protein. This association with a Cullin
protein will further be supported by a conserved Cullin box motif, located downstream of the B/C box in the SOCS box (Kamura et al., 2004). Cullin in turns recruits a RING finger-containing protein Rbx, thereby completing the assembly of the E3 ligase complex (figure 10) (Iwai et al., 1999; Pause et al., 1997).

**Figure 10: Model for proteasomal target degradation by SOCS proteins**

VHL and SOCS1 were shown to interact with a Cul2-Rbx1 module, whereas the other SOCS proteins associate with a Cul5-Rbx2 complex (Kamizono et al., 2001; Kamura et al., 1999). The Cul2 box and Cul5 box were defined as key determinants of the association between Elongin B/C binding proteins and a specific Cullin-Rbx module. The Cul5 box corresponds to the C-terminal portion of the canonical SOCS box and has the consensus sequence ΦxxLPΦPxxΦxx(Y/F)(L/I), where the central LPΦP is particularly important for Cullin5 binding (Hilton et al., 1998; Kamura et al., 2004; Mahrour et al., 2008). Recently, the consensus sequence for the Cul2 box was defined as a ΦPxxΦxxxΦ motif, sharing some sequence similarity with the Cul5 box (Mahrour et al., 2008).
Together with an E1 ubiquitin activating enzyme and the E2 conjugating enzyme, the E3 ubiquitin ligase participates in the polyubiquitin tagging of associated proteins and is responsible for substrate specificity (figure 11) (Glickman and Ciechanover, 2002). This way, SOCS box proteins may suppress signalling by linking associated signalling components for degradation (Kamura et al., 2001). Consistent with this idea, proteasomal inhibitors block the inhibitory functions of SOCS and induce sustained JAK-STAT signalling (Callus and Mathey-Prevot, 1998; Kim and Maniatis, 1996; Verdier et al., 1998; Yu and Burakoff, 1997). The functional importance of the SOCS box was confirmed by the generation of transgenic mice expressing a C-terminal truncated variant of SOCS1 or SOCS3. These mice exhibit impaired regulation of respectively INFγ and G-CSF signalling, suggesting that the SOCS box is essential for complete SOCS activity (Boyle et al., 2007; Zhang et al., 2001).
In this thesis, we studied the multiple roles of the SOCS box. Other functions of this domain are: (i) the control of protein stability, (ii) an adaptor function linking SOCS to other signalling pathways like the MAPK pathway, (iii) an implication in SOCS cross-regulation (chapter 9) and (iv) regulation of receptor interaction (chapter 10). Considering the different characteristics and functions of the SOCS box, a complex biological role for this domain emerges which is the focus of a review (chapter 11).

III. Posttranslational modification of SOCS proteins

Ubiquitination of CIS and SOCS3 was reported (Sasaki et al., 2003; Verdier et al., 1998), as well as phosphorylation of SOCS1 and SOCS3 (see table 9 for overview). One of the tyrosines phosphorylated in the SOCS box of SOCS3 is highly conserved in all SOCS family members. It will thus be important to determine whether other SOCS proteins can also be regulated as a result of phosphorylation.

Table 9: Phosphorylation of SOCS proteins

<table>
<thead>
<tr>
<th>phosphorylation sites</th>
<th>kinase/stimulus</th>
<th>effect</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SOCS1</strong> Ser/Thr in the N-terminus</td>
<td>PIM kinase</td>
<td>stabilisation of SOCS1</td>
<td>(Chen et al., 2002)</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>PIM kinase</td>
<td>potentiates STAT5 inhibition (through SOCS1 stabilisation?)</td>
<td>(Peltola et al., 2004)</td>
</tr>
<tr>
<td><strong>SOCS3</strong> Tyr204 and 221 in the SOCS box</td>
<td>JAK kinase</td>
<td>disrupts Elongin interaction and destabilises SOCS3</td>
<td>(Haan et al., 2003)</td>
</tr>
<tr>
<td>Tyr204 and 221 in the SOCS box</td>
<td>JAK and Src kinases, RTKs (in response to IL-2, EPO, EGF, PDGF)</td>
<td>maintains activation of ERK-MAPK</td>
<td>(Cacalano et al., 2001)</td>
</tr>
<tr>
<td>Tyr</td>
<td>JAK kinase (in response to IL-2)</td>
<td></td>
<td>(Cohney et al., 1999)</td>
</tr>
<tr>
<td>Tyr</td>
<td>JAK kinase (in response to insulin)</td>
<td></td>
<td>(Peraldi et al., 2001)</td>
</tr>
<tr>
<td>Tyr221 in the SOCS box</td>
<td>RTK (in response to PDGFR)</td>
<td>interacts with and regulates Nck and Crk-L adaptors</td>
<td>(Sitko et al., 2004)</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>PIM kinase</td>
<td>potentiates STAT5 inhibition (through SOCS1 stabilisation?)</td>
<td>(Peltola et al., 2004)</td>
</tr>
</tbody>
</table>
IV. Structural determination of SOCS proteins

The first SOCS structure to be resolved was that of SOCS3 (lacking the first 21 AA and the SOCS box) in complex with a phosphotyrosine peptide from the gp130 chain (Babon et al., 2006). This structure revealed the basis for an extended SH2 domain that provides an interaction interface for phosphotyrosine motifs. Also an unstructured PEST (proline-, glutamic acid-, serine and threonine rich) motif in the SH2 domain was identified that negatively regulates SOCS3 protein stability (Babon et al., 2006).

Figure 12: Alternative domain organisation in the SOCS2 and -4 ternary complexes
Comparison of the SOCS2-ElonginB/C and SOCS4-ElonginB/C structures highlighting the switch in packing between the SOCS2/SOCS4 C-terminus and the N-terminal ESS helix (from Bullock et al., 2006; Bullock et al., 2007).

The crystal structures of the ternary complexes of SOCS2 and SOCS4 with Elongin B/C revealed a common tripartite domain structure for SOCS proteins with an N-terminal ESS helix that stabilizes the central SH2 domain and a C-terminal SOCS box that mediates a conserved four helix bundle interaction with Elongin C (figure Chapter 3: The SOCS protein family
12). Nonetheless, two distinct SOCS subclasses were defined that each make alternative use of N- and C-terminal structures to stabilise the SH2-SOCS box interdomain interface (Bullock et al., 2006; Bullock et al., 2007).

Based on the structural information of SOCS2, it was proposed that in SOCS1-3 and CIS the C-terminus is buried in the core of the structure where it stabilizes the interaction between the SH2 domain and the SOCS box. This packing partially exposes the N-terminal ESS providing better accessibility for the SOCS1 and SOCS3 KIR domain. Evidently, this domain organization precludes C-terminal extensions and explains the strictly conserved length of the C-terminal parts in CIS and SOCS1-3 (Bullock et al., 2006). In contrast, the SOCS4-7 subclass contains extended C-termini and there the N-terminal ESS helix is buried in the SOCS box and SH2 interface to fulfil an equivalent packing role as could be demonstrated in the SOCS4 structure. The function of the C-terminus is then redefined to stabilize an interface with the N-terminal domain (Bullock et al., 2007).

As a result of the apparent structural and functional interdependence of the different SOCS domains, studies using domain deletions or mutations of core positions in the interface have to be interpreted with caution as these may lead to a loss of structural integrity. However, structural alterations can act as a physiological control mechanism used to regulate the activity and interplay between SOCS family members. Phosphorylation of the SOCS3 C-terminus will for example prevent its core interaction resulting in loss of Elongin C binding and proteasomal degradation (Haan et al., 2003). In the same line, Elongin B/C association to CIS has a structural impact on SH2 functionality as disruption of this interaction can lead to loss of receptor binding (chapter 10).

V. The physiology of SOCS functions

In vitro studies reveal the potential of SOCS family members to inhibit multiple cytokine induced signalling pathways. Studies based on ectopic expression of SOCS have provided valuable insights into the mechanisms of SOCS functions. But as they rely on overexpression, these studies may overestimate the range of
SOCS actions and have to be interpreted in function of physiological expression levels. Therefore, gene targeting analyses have been used in order to elucidate the physiological actions of SOCS proteins (table 10). Studies in transgenic mice revealed that SOCS have essential roles in the regulation of various cytokines and exert more specific actions than those expected from overexpression studies in vitro (Greenhalgh and Alexander, 2004). Of note, loss-of-function studies can only identify the non-redundant functions of each SOCS protein. It will be interesting to explore whether SOCS have overlapping actions that are not revealed by mice lacking single SOCS genes.

SOCS1

In vitro studies implicated SOCS1 in the inhibition of multiple signalling systems including GH, Epo, PRL, IL-6, IFNα/β, IFNγ and IL-4 (Adams et al., 1998; Dif et al., 2001; Hansen et al., 1999; Song and Shuai, 1998). The potent inhibition and wide range of action exhibited by SOCS1 is most probably due to its ability to inhibit the catalytic activity of JAK kinases. Moreover, SOCS1 induces the ubiquitination and destruction of VAV, JAK2 and the TEL-JAK2 oncogene in a SOCS box dependent fashion (De Sepulveda et al., 2000; Frantsve et al., 2001; Kamizono et al., 2001; Ungureanu et al., 2002). Regulation of NF-κB by ubiquitin-mediated proteolysis of its p65/RelA subunit was also found to be facilitated by SOCS1 (Ryo et al., 2003). Furthermore, SOCS1 (and SOCS3) can promote destruction of Insulin Receptor Substrate (IRS) 1 or IRS2 and focal adhesion kinase (FAK), inhibiting respectively insulin- and FAK-dependent signalling events (Liu et al., 2003; Rui et al., 2002). Despite these observations, in vitro studies have generally failed to prove a requirement of the SOCS box for the inhibitory functions of SOCS1 (Narazaki et al., 1998; Nicholson et al., 1999; Yasukawa et al., 1999). Nevertheless, a clear contribution of this domain was found for the inhibition of in vivo cytokine action. Mice expressing a C-terminal truncated variant of SOCS1 were hyper-responsive to IFNγ and died prematurely due to an inflammatory disease similar to, but less severe than the pathology SOCS1−/− mice (Zhang et al., 2001). This demonstrates that the SOCS box is required for optimal functioning.
SOCS1 knock-out mice die within three weeks after birth with a phenotype characterized by stunted growth, fatty degeneration of the liver, severe lymphopenia, monocytic infiltration of major organs and peripheral T cell activation (Naka et al., 1998; Starr et al., 1998). These multiple deregulations of the immune system were attributed to uncontrolled IFNγ signalling caused by constitutive activation of STAT1. In support of this, the complex disease in SOCS1−/− mice was prevented by administration of neutralizing anti-IFNγ antibodies and did not occur in double knock-out mice also lacking the IFNγ gene (Alexander et al., 1999; Marine et al., 1999b). Anyway, SOCS1 is not restricted to IFNγ actions, as studies in the combined SOCS1−/−IFNγ−/− double knock-out mice revealed lethality at later stages due to a range of inflammatory diseases (Metcalf et al., 2002). Indeed, SOCS1 deficient mice also lacking STAT6 or STAT4 that are downstream effectors of IL-4 or IL-12 signalling, respectively, are rescued from neonatal lethality (Eyles et al., 2002; Naka et al., 2001), suggesting that SOCS1 affects not only IFNγ but also IL-4 and IL-12 signalling in vivo. Further studies of SOCS1−/− mice revealed that SOCS1 regulates signals of TNFα, LPS and insulin (Kawazoe et al., 2001; Kinjyo et al., 2002; Morita et al., 2000; Nakagawa et al., 2002). SOCS1 conditional knock-out mice demonstrated an inhibitory role for SOCS1 on γc cytokines, such as IL-2 or IL-7 (Chong et al., 2003; Cornish et al., 2003). SOCS1 transgenic mice are characterized by defective thymocyte development and perturbed homeostasis of T cells (Fujimoto et al., 2000).

SOCS3

Although SOCS3 is structurally similar to SOCS1, the mechanisms by which these two molecules negatively regulate signalling differ in more than one aspect. Whereas SOCS1 blocks signalling by binding directly to JAK kinases, SOCS3 adequately inhibits activation of JAKs only when bound in close proximity to the kinase. Membrane proximal association of SOCS3 was found for different cytokine receptors including gp130, LR, EpoR, LIFR, IL-6R, IL-12R and GHR (Bjorbaek et al., 2000; Eyckerman et al., 2000; Hortner et al., 2002; Lehmann et al., 2003; Ram and Waxman, 1999; Sasaki et al., 2000; Yamamoto et al., 2003). SOCS3 was also found to promote degradation of target proteins like IRS adaptors, FAK kinase and
Siglec receptors (Liu et al., 2003; Orr et al., 2007a; Orr et al., 2007b; Rui et al., 2002). Deletion of the SOCS box of SOCS3 in transgenic mice leads to impaired regulation of G-CSF signalling and response to inflammatory stimuli, establishing a role for the SOCS box in the in vivo actions of SOCS3 (Boyle et al., 2007).

Table 10: Overview of the phenotypes of SOCS knock-out and transgenic mice

<table>
<thead>
<tr>
<th>SOCS</th>
<th>knock-out phenotype</th>
<th>transgenic phenotype</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOCS1</td>
<td>neonatal lethality due to severe defects in immune system</td>
<td>defects in T cell development</td>
<td>Naka et al., 1998&lt;br&gt;Starr et al., 1998&lt;br&gt;Fujimoto et al., 2000</td>
</tr>
<tr>
<td>SOCS3</td>
<td>embryonic lethality due to placental insufficiency</td>
<td>embryonic lethality due to defective foetal liver erythropoiesis</td>
<td>Marine et al., 1999a&lt;br&gt;Roberts et al., 2001&lt;br&gt;Takahashi et al., 2003</td>
</tr>
<tr>
<td>CIS</td>
<td>no defects</td>
<td>defective in growth, immune responses and mammary gland development</td>
<td>Marine et al., 1999a&lt;br&gt;Matsumoto et al., 1999</td>
</tr>
<tr>
<td>SOCS2</td>
<td>excessive growth</td>
<td>excessive growth</td>
<td>Metcalf et al., 2000&lt;br&gt;Greenhalgh et al., 2002b</td>
</tr>
<tr>
<td>SOCS4</td>
<td>not reported</td>
<td>not reported</td>
<td></td>
</tr>
<tr>
<td>SOCS5</td>
<td>no defects</td>
<td>altered Th1/Th2 cell balance</td>
<td>Seki et al., 2002&lt;br&gt;Brender et al., 2004</td>
</tr>
<tr>
<td>SOCS6</td>
<td>mild growth retardation</td>
<td>improved insulin and glucose tolerance</td>
<td>Krebs et al., 2002&lt;br&gt;Li et al., 2004</td>
</tr>
<tr>
<td>SOCS7</td>
<td>high lethality due to hydrocephalus</td>
<td>not reported</td>
<td>Krebs et al., 2004</td>
</tr>
</tbody>
</table>

Both SOCS3 knock-out mice and transgenic mice die in utero. Lack of SOCS3 results in placental insufficiencies caused by uncontrolled LIF signalling, while SOCS3 overexpression results in defective foetal liver erythropoiesis (Marine et al., 1999a; Roberts et al., 2001; Takahashi et al., 2003). Studies in conditional SOCS3 knock-out mice demonstrated that SOCS3 is an important negative regulator of G-CSF (Croker et al., 2004; Kimura et al., 2004) and IL-6 (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003). As mentioned before, SOCS3 not only controls the magnitude of IL-6 signalling but also shapes its responses. Upon deletion of SOCS3 in macrophages IL-6 loses its pro-inflammatory function but elicits STAT3-mediated immunosuppressive actions, similar to IL-10 (Yasukawa et al., 2003). Furthermore, IL-6 also strongly activates STAT1, thereby inducing the expression of

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IFN-responsive genes in these SOCS3 deficient macrophages (Croker et al., 2003; Lang et al., 2003). Together, these data suggest that SOCS3 is a negative regulator of the gp130 family of cytokine receptors in vivo. Essential roles for SOCS3 in the endocrine system like in leptin and insulin signalling have also been identified (Bjorbaek et al., 1998; Emanuelli et al., 2000). Consequently, SOCS3 was reported to be involved in leptin and insulin resistance associated with respectively obesity and type II diabetes (see below) (Howard et al., 2004; Mori et al., 2004; Ueki et al., 2004).

CIS

CIS is induced by cytokines that activate STAT5, such as Epo, GH, PRL, IL-2 and IL-3 and was reported to subsequently suppress these particular signalling cascades by masking the STAT5 binding sites in their receptors (Aman et al., 1999; Dif et al., 2001; Endo et al., 2003; Hansen et al., 1996; Matsumoto et al., 1997; Ram and Waxman, 1999; Verdier et al., 1998; Yoshimura et al., 1995). Based on the non-overlapping binding pattern, direct competition of CIS with STAT5 for common phosphotyrosine binding sites was recently excluded for the GHR (Uyttendaele et al., 2007). Also at the G-CSFR CIS does not compete with STAT5 for binding to receptor motifs, reflecting a reliance on other inhibitory mechanisms. Indeed, CIS association was found to induce proteasome-dependent degradation of the EpoR and GHR and in the latter case, CIS also was reported to play a role in GHR internalization (Landsman and Waxman, 2005; Ram and Waxman, 2000; Verdier et al., 1998).

Mice deficient for CIS expression are phenotypically normal, perhaps due to functional compensation by other SOCS proteins (Marine et al., 1999a). CIS transgenic mice exhibit growth retardation, impaired mammary gland development and immune defects like reduced numbers of natural killer (NK) and NK T cells (Matsumoto et al., 1999). These phenotypes are remarkably similar to those observed in STAT5a and/or STAT5b knock-out mice, lending support for CIS as a specific negative regulator of STAT5-mediated cytokine signalling (Matsumoto et al., 1999; Teglund et al., 1998).
The mechanism by which SOCS2 exerts its regulatory functions remains more elusive. SOCS2 can probably attenuate signalling in a similar way as CIS, involving competition at receptor sites. Interaction of SOCS2 with phosphotyrosine motifs on the GHR, PRLR, EGFR, EpoR and LR has been reported (Eyckerman et al., 2001; Goldshmit et al., 2004; Greenhalgh et al., 2005; Lavens et al., 2006; Pezet et al., 1999). Receptor association of CIS and SOCS2 are studied in more detail in part II. SOCS2 might act as an ubiquitin ligase as the SOCS box of SOCS2 appeared to be crucial for the negative regulation of GH signalling (Greenhalgh et al., 2005). Furthermore, the solving of the SOCS2-Elongin B/C crystal structure revealed a prototypical SOCS box ubiquitin ligase architecture (Bullock et al., 2006), further supporting a role for SOCS2 in E3 ligase activity.

Initial in vitro studies reported a dual effect of SOCS2 in GH signalling: low SOCS2 concentrations moderately inhibit GH signalling while higher levels positively regulate signalling by blocking the inhibitory effects of other SOCS proteins (Favre et al., 1999). Interference of SOCS2 with other SOCS proteins was observed in several cytokine receptor systems including PRL (Dif et al., 2001; Pezet et al., 1999), IL-2 and IL-3 (Tannahill et al., 2005), IFN type I and leptin signalling (Lavens et al., 2006; Piessevaux et al., 2006). A positive role for SOCS2 was also proposed in mesenchymal precursor cells where it could potentiate osteoblast differentiation through upregulation of JunB expression, possibly through its negative effect on other SOCS proteins (Ouyang et al., 2006). While SOCS1 and SOCS3 are typically induced in a rapid and transient manner upon receptor activation, expression of SOCS2 usually occurs later after cytokine stimulation and is more prolonged (Adams et al., 1998; Brender et al., 2001; Pezet et al., 1999; Rico-Bautista et al., 2004; Tannahill et al., 2005; Tollet-Egnell et al., 1999). This is in line with the cross-regulatory potential of SOCS2 that can downregulate expression of other SOCS molecules and restore cellular sensitivity (discussed in chapter 9).

Mice lacking SOCS2 exhibit gigantism associated with increases in bone and body length and enhanced weight of organs and carcass (Metcalf et al., 2000). This phenotype is due to prolonged STAT5 signalling in response to GH stimulation.
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SOCS2

SOCS2−/− STAT5b−/− double knock-out mice showed normal growth (Greenhalgh et al., 2002a), suggesting that SOCS2 is involved in negative regulation of the GH signalling pathway. SOCS2 transgenic mice also grew significantly larger than their wild type littermates, further supporting a dual role for SOCS2 (Greenhalgh et al., 2002b).

SOCS4

The remaining SOCS members, SOCS4-SOCS7, which represent the direct orthologs of ancestral SOCS family members, have been poorly examined. Especially about SOCS4 very little is known concerning expression and molecular mode of action. It was reported that Mycobacterium tuberculosis infection in mice is correlated with augmented levels of type IFNs, SOCS4 (and SOCS5) (Manca et al., 2005), suggesting a role in immunity. SOCS4 (and its closest homolog SOCS5) are upregulated upon epidermal growth factor (EGF) stimulation and markedly reduce EGFR levels thereby inhibiting the mitogenic signalling (Kario et al., 2005). The STAT3 binding site in EGFR was identified as a high affinity SOCS4 substrate and the structural resolution of the SOCS4-Elongin B/C complex defined a molecular basis for SOCS mediated EGFR degradation (Bullock et al., 2007). No SOCS4 knock-out or transgenic mice are reported, leaving the biological role of this SOCS member unclear.

SOCS5

SOCS5 is expressed in many tissues and especially in hematopoietic tissues (Magrangeas et al., 2000). As mentioned above SOCS5 is induced upon EGF stimulation and subsequently promotes SOCS box dependent turnover of EGFR (Kario et al., 2005; Nicholson et al., 2005). Accordingly, the Drosophila SOCS36E, highly similar to mammalian SOCS5, was found to temper EGF responses (Callus and Mathey-Prevot, 2002). SOCS5 was also demonstrated to mildly suppress IL-6 and LIF-induced signalling (Nicholson et al., 1999) and to promote Th1 differentiation by inhibiting STAT6 dependent IL-4 signalling (Seki et al., 2002).
Accordingly, T cells from transgenic mice constitutively expressing SOCS5 exhibited a significant reduction of IL-4 mediated Th2 development. Unexpectedly, no abnormalities in Th1/Th2 differentiation were found in SOCS5 deficient T cells and SOCS5 knock-out mice showed normal susceptibility to pathogen infections (Brender et al., 2004). This may be explained by SOCS5 being compensated for by other SOCS proteins such as SOCS4, as SOCS4 shares significant identity with SOCS5. Analyses of SOCS4−/−SOCS5−/− double knockout mice will be required to address the function of SOCS5 in vivo.

SOCS6

SOCS6 is induced by insulin and subsequently interacts with the insulin receptor (Li et al., 2004; Mooney et al., 2001). This association may occur indirectly since SOCS6 binding with signal transducers including IRS-2, IRS-4 and the p85 regulatory subunit of PI-3K, was reported in response to insulin stimulation (as well as IGF-1 stimulation) (Krebs et al., 2002). SOCS6 expression can also be upregulated by stem cell factor (SCF) and the interaction of SOCS6 with the KIT receptor upon SCF stimulation was shown to affect upstream signalling components leading to MAPK activation (Bayle et al., 2004). After insulin or SCF stimulation, an inhibitory effect is observed on ERK1/2 stimulation but not on Akt activation (Bayle et al., 2004; Li et al., 2004; Mooney et al., 2001). SOCS6 may even have a positive effect on activation of Akt upon insulin stimulation, possibly due to binding to the p85 subunit of PI3K, thereby overcoming the negative effects that p85 monomers have on PI-3K signalling (Li et al., 2004). SOCS6 was found to associate with the leptin receptor (Montoye et al., 2006). It also interacts with haem-oxidized IRP2 ubiquitin ligase 1 (HOIL1), driving ubiquitination and degradation of proteins associated with SOCS6 (Bayle et al., 2006). Recently, SOCS6 was proposed to negatively regulate STAT3 protein levels and a nuclear function for SOCS6 was proposed, depending on its N-terminus (Hwang et al., 2007).

SOCS6 deficient mice develop normally and exhibit no defects in haematopoiesis or glucose homeostasis. However, they weighed approximately 10% less than wild-type littermates (Krebs et al., 2002). It was suggested that the closely related
SOCS7 could functionally compensate for SOCS6 deficiency. By contrast, SOCS6 transgenic mice displayed improvement in insulin signalling and glucose metabolism (Li et al., 2004).

**SOCS7**

In contrast to other SOCS proteins, the N-terminal domain of SOCS7 contains several recognizable motifs: multiple poly-proline motifs, which are possible docking sites for SH3 domain containing proteins and a putative nuclear localisation signal (NLS), indicating a potential role for SOCS7 in the nucleus (Matuoka et al., 1997). The N-terminus of SOCS7 mediates interactions with the cytoskeletal protein vinexin and several signalling molecules like PLCγ, Grb2 and Nck, which are involved in signalling mediated by a large number of receptors (Martens et al., 2004; Matuoka et al., 1997). SH2 dependent interactions of SOCS7 were demonstrated with the EGFR and LR and several components of the insulin pathway including IRS1, IRS2, IRS4, p85 and the insulin receptor (Banks et al., 2005; Krebs et al., 2002; Matuoka et al., 1997; Montoye et al., 2006). SOCS7 can suppress PRL, GH and leptin signalling by interacting with STAT5 or STAT3 and attenuating their nuclear translocation (Martens et al., 2005).

SOCS7 deficient mice exhibit no defects in haematopoiesis or circulating glucose or insulin concentrations but they are smaller than their wild-type littermates (Krebs et al., 2004). The pancreatic islets of Langerhans are in some cases exceptionally large (Banks et al., 2005; Krebs et al., 2004). Within 15 weeks however, about 50% of the SOCS7 knock-outs died as a result of hydrocephalus (Krebs et al., 2004). Accordingly, prominent expression of SOCS7 was found in the brain, suggestive of an important functional role of SOCS7 in this organ (Banks et al., 2005; Krebs et al., 2002). Older mice also develop increased glucose tolerance and insulin sensitivity together with a mild hyperglycaemia and hyperinsulinemia (Banks et al., 2005). Although deficiency of SOCS6 or SOCS7 in mice results in relatively mild phenotypes at birth (Krebs et al., 2004; Krebs et al., 2002), the double knock-out animals are embryonically lethal, perhaps pointing to the loss of redundant functions of SOCS6 and SOCS7 (Dr. Hilton, personal communication).
SOCS proteins and immunity

Cytokines mediate communication between cells of the immune system and a tight control of cytokine receptor signalling is of crucial importance to balance antimicrobial and tissue-destructive effects. Mainly SOCS1 and SOCS3 are critical mediators of both innate and adaptive immunity through the regulation of cytokine signalling in T cells and antigen-presenting cells, including macrophages and dendritic cells (DCs).

SOCS in innate immunity - Toll-like receptor (TLR) signalling that initiates innate immune responses to pathogens, induces expression of SOCS proteins (Baetz et al., 2004; Dalpke et al., 2001; Naka et al., 2005; Stoiber et al., 1999). SOCS1-deficient mice are highly sensitive to sepsis induced by lipopolysaccharide (LPS), a known TLR activator, and endotoxin tolerance was absent. Additionally, macrophages, DCs and fibroblasts from SOCS1-/- mice produce increased levels of pro-inflammatory cytokines such as TNF, IL-12 and IFNγ, as well as nitric oxide, in response to TLR ligands (Chinen et al., 2006; Hanada et al., 2005; Kinjyo et al., 2002; Nakagawa et al., 2002). A direct effect of SOCS1 on the TLR pathway has been proposed as SOCS1 was found to bind to the p65 subunit of NF-κB and promotes its turnover (Ryo et al., 2003). SOCS1 also mediates ubiquitination and degradation of the tyrosine phosphorylated TLR adaptor MyD88 Adaptor-Like (Mal), thereby leading to the suppression of Mal-dependent p65 phosphorylation and transactivation of NF-κB (Mansell et al., 2006). In addition to the NF-κB pathway, SOCS1 might also target the JUN N-terminal kinase (JNK) and p38 MAPK cascades by inducing the degradation of the upstream activator apoptosis signal-regulating kinase 1 (ASK1) (He et al., 2006). Other groups reported that the principal mode of suppression of SOCS1 is the inhibition of the secondary activated type I IFN signalling pathway (Baetz et al., 2004; Gingras et al., 2004). The induction of SOCS1 by chemotactic factors such as fMLP and IL-8 provides evidence for SOCS mediated cross-talk between chemoattractants and cytokine signalling pathways (Johnston, 2004).
SOCS3 is a key determinant for regulating and shaping the divergent activities of IL-6 and IL-10 in macrophages following TLR stimulation (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003). SOCS2 was found to be crucial for the anti-inflammatory effects of lipoxins, although the molecular mechanism remains elusive (Machado et al., 2006).

**SOCS in adaptive immunity** - SOCS proteins are crucially involved in T-helper cell differentiation and T and B cell responses of the adaptive immunity. This is well illustrated by the SOCS1\(^{-/-}\) mice in which those aspects are massively disturbed (Alexander et al., 1999; Chong et al., 2003; Eyles et al., 2002; Fujimoto et al., 2002; Marine et al., 1999b; Naka et al., 1998). The pivotal role of SOCS1 in Th1 differentiation and inhibition of IFN\(\gamma\) and other cytokines involved in lymphocyte homeostasis, such as IL-2, IL-4, IL-7 and IL-12 appeared from studies in knock-out mice (see above) (Eyles et al., 2002; Naka et al., 2001). More specific effects have been reported for SOCS family members such as SOCS5 that has the capacity to impair IL-4 mediated Th2 differentiation and thus promote Th1 cell differentiation (Seki et al., 2002). SOCS3 displays an expression pattern reciprocal to that of SOCS1 and SOCS5 and is exclusively associated with Th2 cell differentiation (Egwuagu et al., 2002; Li et al., 2006).

CD25(+)CD4(+) regulatory T cells (Tregs) are actively engaged in the maintenance of immunologic self-tolerance by suppressing the autoreactive responses mediated by effector T cell functions. SOCS1, SOCS2 and CIS are highly induced in activated CD4(+)CD25(+) Tregs (McHugh et al., 2002). In a DNA microarray analysis, performed to identify Treg-specific molecules controlled by the transcription factor Foxp3, SOCS2 was one of the predominantly expressed genes (Sugimoto et al., 2006). SOCS1 was reported to prevent the development of dextran sulfate sodium (DSS)-induced colitis (a model of colitis resembling human IBD) by inhibiting IFN\(\gamma\)/STAT1 signalling and by subsequently regulating Treg cell development (Horino et al., 2008). Elevated SOCS levels in Tregs may be required to strictly control their immunosuppressive functions to achieve a balance between the necessity to suppress autoreactivity and the ability to allow appropriate responses to foreign antigens.
VI. **SOCS proteins in disease**

Through their impact on cytokine- and growth factor-activated signalling pathways, it seems inevitable that disruption of normal SOCS function will contribute to disease onset and progression. Accordingly, therapeutic strategies based on the manipulation of SOCS activity might be of clinical benefit.

*Infectious disease pathogenesis (hijacking the host’s SOCS system)*

Interfering with SOCS regulation of cytokine signalling is an effective strategy used by various microbial pathogens to manipulate cytokine signalling and as a result escape detrimental immune responses. The parasites *Toxoplasma gondii* and *Leishmania* can for instance induce host SOCS expression to evade immune responses (Alexander et al., 1999; Mun et al., 2005; Zimmermann et al., 2006). Also, bacteria such as *Mycobacteria*, *Salmonella typhimurium* and *Listeria pseudomallei*, are able to induce endogenous SOCS and consequently exploit these host negative regulatory mechanisms for their own purpose (Blumenthal et al., 2005; Manca et al., 2005; Stoiber et al., 2001; Uchiya and Nikai, 2005; Vazquez et al., 2006). Finally, viruses will also target host immunity by misusage of endogenous SOCS proteins. Hepatitis C Virus (HCV) core protein was reported to impair IFNα-induced signal transduction via SOCS3 expression in hepatic cells (Bode et al., 2003). Human immunodeficiency virus (HIV) induces SOCS expression known to interfere with Th differentiation and Ig class switching, thereby promoting HIV infection (Moutsopoulos et al., 2006; Qiao et al., 2006). Recently, SOCS1 was reported to associate with the HIV-1 p55 Gag polyprotein to enhance its stability and trafficking, resulting in the efficient production of HIV-1 particles (Ryo et al., 2008).

It is of note that, similar to SOCS molecules, some viral proteins can act as the substrate recognition unit of an E3 ligase complex, targeting host proteins for ubiquitination and proteasomal degradation. In this respect, HIV-1 encoded viral infectivity factor (Vif) associates with an ElonginB/C-Cullin 5 module in order to direct the turnover of APOBEC3G, a host factor that induces hypermutations in newly synthesized viral DNA (Rose et al., 2004). Other examples of viral proteins...
that assemble ECS-based E3 ligases are the Respiratory Syncytial Virus NS1 and the adenovirus E4orf6 that respectively target STAT2 and p53 for proteasomal destruction (Elliott et al., 2007; Luo et al., 2007). Possibly, this sequestering of E3 components and especially Elongin B/C, will further deregulate the host SOCS actions.

Targeting SOCS proteins for improving the defense against pathogens might thus be a beneficial approach in case of infection. Nevertheless, therapeutic modulation of SOCS will have to be done cautiously since inhibition of SOCS to counter microbial infection may also lead to an enhanced immune response associated with pro-inflammatory effects.

Inflammatory diseases

SOCS proteins are crucially implicated in the regulation of JAK/STAT signalling in inflammation. Accordingly, alterations in SOCS protein levels, and more specific SOCS3, has been associated with the pathogenesis of various inflammatory diseases including rheumatoid arthritis (RA), Crohn’s disease and inflammatory bowel diseases (IBD) (Egan et al., 2003; Isomaki et al., 2007; Lovato et al., 2003; Rakoff-Nahoum et al., 2006; Suzuki et al., 2001). SOCS3 expression is induced by a wide variety of inflammatory and anti-inflammatory cytokines, including IFNγ, IL-3, IL-6 and IL-10 and will function to counteract STAT3 activation associated with inflammatory disorders. Additionally, exogenous SOCS3 delivery was proven to be an effective therapy to attenuate inflammation as was shown in different models including mice with experimental induced colitis and arthritis (Fang et al., 2005; Jo et al., 2005; Shouda et al., 2001; Suzuki et al., 2001). SOCS3 was also demonstrated to have an important role in regulating the onset and maintenance of Th2 mediated allergic immune diseases. Enhanced SOCS3 levels in T cells have for example been associated with asthma pathogenesis (Seki et al., 2003). Humans with allergic conjunctivitis showed a correlation between the level of expression of SOCS3 and the severity of the disease (Ozaki et al., 2005; Seki et al., 2003). A similar role for SOCS5 has also been reported in a mouse model of this disease (Ozaki et al., 2005), as well as in murine experimental autoimmune uveitis, an autoimmune disease of the retina (Takase et al., 2005). Single nuclear polymorphisms (SNPs) in
SOCS1 that result in SOCS1 upregulation in T cells have been associated with asthma pathogenesis (Harada et al., 2007). Clearly, therapeutical targeting of SOCS might attenuate inflammatory cytokine circuits in autoimmune and other inflammatory diseases. Modulation of SOCS expression or function will also be an important therapeutic strategy for immunological diseases induced by an abnormal Th1/Th2 balance.

Metabolic diseases (leptin and insulin resistance)

Different observations put SOCS3 forward as a crucial inhibitor of LR signalling and suggest a prominent role for increased SOCS3 levels in leptin resistance and consequent obesity (Bjorbaek et al., 1998; Dunn et al., 2005; Howard et al., 2004; Mori et al., 2004). SOCS factors are critically implicated in attenuation of insulin signalling and SOCS1 and SOCS3 might be players in the development of insulin resistance associated with type 2 diabetes (Emanuelli et al., 2001; Emanuelli et al., 2000; Kawazoe et al., 2001; Ueki et al., 2005; Ueki et al., 2004). Some evidence also points to SOCS2 as a modulator of insulin signalling (Rico-Bautista et al., 2006) and interestingly, the prevalence of SNPs in the SOCS2 gene was correlated with type 2 diabetes (Kato et al., 2006). Additionally, it was suggested that aberrations in the ubiquitin-proteasome pathway might as well be one of the molecular mechanism behind insulin resistance through inappropriate degradation of IRS1 or IRS2 by SOCS proteins (Balasubramanyam et al., 2005). It can be envisaged that blocking SOCS actions can be used clinically to treat leptin or insulin resistance, and accordingly SOCS3 might be a potential therapeutic target for prevalent human metabolic disorders such as obesity and diabetes.

Growth related pathologies

Haplotype insufficiency for SOCS2 promotes trophic actions of GH in small intestine and promotes preneoplastic growth in colon during excess GH. Small variations in SOCS2 expression levels may thus significantly influence the outcome of therapeutic GH or acromegaly in intestine (Michaylira et al., 2006). Hepatic growth
hormone resistance during sepsis is associated with increased SOCS expression and impaired growth hormone signalling (Yumet et al., 2006). Pharmacological targeting of specific negative regulators of growth signalling, like SOCS2, may be valuable in the development of novel therapies targeting growth disorders. Moreover, it may have the potential to enhance the beneficial actions of GH in growth and metabolism, without the side effects associated with direct GH treatment.

Cancers and haematopoietic disorders

Several oncologic disorders are associated with enhanced JAK-STAT activity, promoting cell proliferation and survival which will contribute to malignant growth (Chai et al., 1997; Frank et al., 1997; Gouilleux-Gruart et al., 1996). Inactivation of SOCS by gene mutation/deletion or reduced SOCS expression due to silencing by DNA hypermethylation are frequently found in hepatocellular, pancreatic, lung, ovarian and breast carcinomas (Farabegoli et al., 2005; He et al., 2003; Komazaki et al., 2004; Nagai et al., 2003; Wikman et al., 2002; Yoshikawa et al., 2001). Compellingly, constitutive activation of the JAK-STAT pathway in several haematological malignancies including leukemia and lymphoma was also associated with SOCS downregulation (Galm et al., 2003; Melzner et al., 2005; Watanabe et al., 2004; Weniger et al., 2006). SOCS proteins appear to have tumor suppressor functions that need to be bypassed for transformation to occur. This implies that the forced expression of SOCS might be beneficial for the treatment of some malignancies.

However, tumor cells upregulating or constitutively expressing SOCS have also been described (Arany et al., 2001; Evans et al., 2007; Faderl et al., 2003; Haffner et al., 2007; Hakansson et al., 2008; Huang et al., 2007; Raccurt et al., 2003; Roman-Gomez et al., 2004) and this may be indicative of a tumor protecting function for SOCS proteins. The in vivo elevation of SOCS gene expression will confer resistance to some host cytokines and may be part of the host/tumour response. In this respect, constitutive SOCS3 expression was found to grant a growth advantage to human melanoma cells by inducing resistance towards the
growth inhibitory effects of cytokines like IL-6 and oncostatin M (OSM) (Komyod et al., 2007). A therapeutic consequence is that anti-tumoral therapy with IFN may fail in some cancers due to constitutive SOCS1 or SOCS3 expression levels (Fojtova et al., 2007; Roman-Gomez et al., 2004; Sakai et al., 2002).

In the case of SOCS2, increased expression levels may enhance sensitivity to cytokine signalling by overcoming the inhibitory effects of other SOCS molecules, thereby contributing to the constitutive active phenotype related to oncogenesis. It was proposed that the increased JAK activity in VHL-mediated renal cell carcinoma (RCC) may be due to SOCS2 recruiting SOCS1 for proteasomal destruction (Wu et al., 2007). SOCS2 expression was enhanced by several cytokines and hormones in leukemic leukocytes (Dogusan et al., 2000). Furthermore, SOCS2 overexpression was clearly found to correlate with advanced stages of chronic myeloid leukemia (CML) (Schultheis et al., 2002; Zheng et al., 2006) or acute myeloid leukemia (AML) (personal communication, Dr. I Touw) (Faderl et al., 2003). This upregulation can be abrogated by STI571, a compound that inhibits the activity of the BCR-ABL tyrosine kinase, which is fundamental for CML pathogenesis (Schultheis et al., 2002). Surprisingly, no haematologic abnormalities were observed in SOCS2 deficient mice. However, as SOCS2 is clearly involved in the development of leukemia, it is conceivable that in the haematopoietic system aberrant SOCS2 upregulation rather than a deficient expression is the important pathologic determinant. As Elongin C overexpression was observed in a number of prostate and breast cancer cell lines (Porkka et al., 2002), this could also possibly lead to a deregulation of signalling via its effect on SOCS stability and functionality.

Some transformation processes result from aberrant SOCS phosphorylation thereby hindering the assembly of the E3 ligase complex and consequently allowing JAKs to evade SOCS regulation. In this context, the JAK2V617F mutant that is associated with myeloproliferative disorders has been demonstrated to evade negative regulation due to hyperphosphorylation of SOCS3. This modification extends SOCS3 half-life time but renders it unable to suppress the activity of the mutant kinase and even stabilizes the mutant JAK, thus potentiating its myeloproliferative capacity (Hookham et al., 2007). Another example concerns the cytokine independent Ser/Thr phosphorylation of SOCS1 by v-Abl in transformed pre-B cells.

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This will disrupt the interaction with Elongin B/C, thereby stabilizing SOCS1 expression and blocking JAK degradation, which will contribute to the transformation process (Limnander et al., 2004).

**The therapeutic applications of SOCS**

As mentioned above, therapeutic application of SOCS proteins could be valuable for the treatment of diseases in which overshoot cytokine signaling is involved, such as those related with inflammation or cancer. One approach would be the overexpression of SOCS molecules. In this respect, the adenovirus-mediated administration of SOCS3 was demonstrated to prevent the development of RA in experimental mouse models (Shouda et al., 2001). Fusion of SOCS proteins with membrane-permeable peptides might be an alternative way to introduce them into cells. In this context, intracellular delivery of a cell-penetrating form of SOCS3 was proven to be effective for treatment of various types of inflammation and septic shock (Jo et al., 2005). A second approach is the generation of small-molecule mimetics of SOCS proteins. The tyrosine kinase inhibitor peptide (TKIP) is an example of a mimetic of SOCS1 and effectively inhibits JAK2-mediated phosphorylation of STATs. This peptide prevented the development of experimental allergic encephalomyelitis (EAE) in mice (Mujtaba et al., 2005) and blocked the proliferation of prostate cancer cell lines (Flowers et al., 2005). A third approach might be blocking SOCS degradation in vivo.

On the contrary, inhibiting SOCS effects could be beneficial in case cytokine action needs to be enhanced. This may be useful for enhancing anti-tumoral or anti-viral immunity, modulating the Th1/Th2 balance, promoting GH signaling or blocking SOCS-mediated resistance against cytokine therapies. Neutralization of SOCS functions could be achieved by the use of their dominant-negative forms. SOCS gene expression could also be silenced by a siRNA or antisense oligonucleotide approach or by the use of specific transcriptional inhibitors. Administration of SOCS1 siRNA by a nanotube carrier could for instance retard the growth of B16 tumours in mice (Yang et al., 2006). siRNA based SOCS1 silencing in DCs elicit HIV-specific T cell and antibody responses, which may open an alternative
possibility for the development of effective HIV vaccines (Song et al., 2006). Another example is the downregulation of SOCS1 and SOCS3 expression in livers of obese diabetic mice by antisense RNA therapy that improved insulin sensitivity and ameliorated hepatic steatosis and hypertriglyceridemia (Ueki et al., 2005). Alternatively, the structural insights in SOCS interactions may provide a basis for the design of SOCS inhibitors.
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CHAPTER 4: MAPPIT, a mammalian two-hybrid system

I. Techniques to study protein-protein interactions

Interactions between proteins form the basis of practically all cellular processes. Protein complexes can form stable structures such as the cytoskeleton or the proteasome complex. On the other hand, protein-protein interactions are also involved in the regulation of cellular processes and these interactions are often temporary and dependent on the modification of one or more of the binding partners. Identification of the interaction profile of a given protein can contribute considerably to its functional characterization in a specific cellular context. Mutations in proteins may cause altered protein-protein interactions, which can result in the onset of diseases. Hence, the identification and manipulation of such protein-protein interactions can offer new strategies towards therapeutic interventions. For this reason, a broad range of both biochemical and genetic methods for studying protein-protein interactions has been developed.

Biochemical approaches

Biochemical methods such as protein affinity chromatography and co-immunoprecipitation aim at isolating protein complexes from cell lysates or from solution. Initially, identification of binding partners by these methods was limited to immunological detection and Edman degradation. However, the introduction of sensitive protein identification by mass spectrometry in combination with database searches led to the development of high-throughput biochemical techniques. A widely used large-scale approach relying on tag-based affinity purification is the tandem affinity purification (TAP) procedure (Puig et al., 2001; Rigaut et al., 1999). Following cellular expression of a bait protein linked to a dual affinity-tag and cell lysis, the protein complexes are precipitated in a two-stage purification step. Proteins are then separated by gel electrophoresis and subsequently identified by
mass spectrometry. This technique has been successfully used to identify yeast, plant as well as mammalian protein complexes (Bouwmeester et al., 2004; Gavin et al., 2002). Luminescence-based mammalian interactome mapping (LUMIER) is an automated high-throughput technology, to map protein-protein interaction networks systematically in mammalian cells (Barrios-Rodiles et al., 2005). The strategy uses Renilla luciferase enzyme fused to proteins of interest, which are then coexpressed with individual Flag-tagged partners in mammalian cells. The interactions are determined by performing a luciferase enzymatic assay upon immunoprecipitation using an antibody against Flag. A limitation of these techniques is that the lysis step may cause disruption of weak interactions. Another large-scale technology relies on protein micro-array chips. High-throughput screening of binary interactions is done by covalently linking proteins to a solid support and screening with fluorescently labelled protein-probes (MacBeath and Schreiber, 2000; Zhu et al., 2001).

Methods for characterization of known interactions include: analytical ultracentrifugation, isothermal titration calorimetry (ITC) and Biacore. This latter technique relies on surface plasmon resonance (SPR), which is an optical phenomenon that occurs when surface plasmon waves are excited at a metal/liquid interface. Biacore allows real time measurement of binding kinetics between two or more molecules. They do so by monitoring the changes in refractive index at the surface layer of a sensor chip, which results from the interaction between surface immobilized and solution-borne binding partners.

*Genetic approaches*

Genetic approaches are in vivo techniques that rely on hybrid bait and prey proteins designed in such a way that their interaction will generate a detectable signal. The yeast two-hybrid method is based on reconstitution of a transcription factor (Fields and Song, 1989). Bait and prey are genetically fused to either the DNA binding domain or the transcription activation domain of the transcription factor. Interaction of bait and prey protein restores transcriptional activity, leading to induction of reporter genes or selection markers. The yeast two-hybrid method has become the most widely used genetic technique, mainly because it is cost-effective, relatively
easy to implement and scalable. A proteome-wide interactome has for example been generated for yeast and C. elegans (Ito et al., 2001; Reboul et al., 2003) and a human interaction map is on its way (Rual et al., 2005; Stelzl et al., 2005). However, although the yeast two-hybrid method is thus far the only approach capable of proteome-wide analyses, the method also suffers from some intrinsic limitations. As functional complementation must occur in the nucleus, failure in nuclear localisation of either bait or prey results in false negatives. Moreover, correct posttranslational modifications, often essential in eukaryotic signalling transduction processes, are hard to reproduce in yeast. Some of the shortcomings described for the yeast two-hybrid system, can be overcome by the use of mammalian cell systems.

The first mammalian two-hybrid methods were mere adaptations of the yeast two-hybrid system. They make use of a similar transcription factor complementation strategy and are accordingly dependent on nuclear localisation of bait and prey (Dang et al., 1991). Several other mammalian systems have been described allowing detection of protein interactions in their physiological context. These methods include the mammalian Ras recruitment system, split-ubiquitin approaches, protein-splicing based assays, reporter enzyme fragment complementation systems, proximity-ligation in situ assay (P-LISA) and MAPPIT (reviewed in (Eyckerman and Tavernier, 2002; Lievens and Tavernier, 2006)). The MAPPIT technology will be described in more detail in the next section. P-LISA allows the study of endogenous protein complexes in intact cells (Soderberg et al., 2006). The technique makes use of oligonucleotides attached to antibodies against the two target proteins that will direct the formation of circular DNA strands when bound in close proximity. The DNA circles in turn serve as templates for localized rolling-circle amplification (RCA). The RCA reaction product is a single-stranded DNA molecule that remains attached to the antibody-protein complex, and is detected through hybridization of a fluorescently labeled oligonucleotide, allowing individual interacting pairs of protein molecules to be visualized.

To visualise protein interactions in real time, methods like fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) or bimolecular fluorescence complementation (BiFC) can be applied. The FRET
technique depends on the energy transfer between two different fluorophores to monitor the interaction and dissociation of the attached proteins. Light emission of the excited donor fluorophore results in excitation of the acceptor fluorophore only when both are brought in close proximity by their fusion partners (Jares-Erijman and Jovin, 2003; Wallrabe and Periasamy, 2005). In the BRET system, excitation of the acceptor fluorophore is induced via enzymatic bioluminescence instead of a donor fluorescence (Angers et al., 2000). In the case of BiFC the target proteins are fused to fragments of a single fluorescent protein, which results in functional complementation upon protein interaction (Hu et al., 2002; Kerppola, 2006).

II. Mammalian protein-protein interaction trap (MAPPIT)

MAPPIT is a mammalian two hybrid technique that was developed in our laboratory and is based on type I cytokine receptor signalling (Eyckerman et al., 2001). In the MAPPIT system (figure 13), a bait protein is coupled C-terminally to a chimeric receptor consisting of the extracellular part of the EpoR and the transmembrane and intracellular parts of the LR that has been deprived of its STAT3 recruiting tyrosine (Y1138) to prevent STAT activation. The other LR tyrosines (Y985 and Y1077) are also mutated to prevent negative feedback, thereby maximizing the signal intensity. MAPPIT prey proteins are linked to a part of the cytoplasmatic tail of the gp130 receptor carrying several STAT3 recruitment sites. When bait and prey interact, phosphorylation of the prey gp130 tail leads to STAT3 recruitment, activation and migration to the nucleus, ultimately resulting in transcription of a reporter gene under the control of the STAT3-responsive rPAP1 promoter.

The MAPPIT technique offers several advantages: first, the mammalian cell context provides a physiological background for the study of posttranslational-dependent interactions. Intrinsic to the strategy, both modification-independent and tyrosine phosphorylation-dependent interactions can be detected. MAPPIT is therefore very suitable for studying protein-protein interactions involved in signal transduction. Second, the physical separation of the bait-prey interaction (cytosol) and the signal read-out by endogenous STATs (nucleus), avoids interference of the chimeric bait
and prey proteins with reporter activity, a common drawback in many two-hybrid methods leading to background signals. Third, MAPPIT is an inducible system based on cytokine stimulation which allows exclusion of ligand-independent interactions, further limiting false positives.

**Figure 13: Principle of MAPPIT**

(A) Schematic representation of the JAK/STAT pathway. (B) MAPPIT, for details see text.

Several variants of the basic MAPPIT approach have been developed. The LR-based MAPPIT, GGS-MAPPIT and $\beta_c$-MAPPIT were used during this thesis (figure 14). The LR-MAPPIT variant was generated to specifically identify interaction partners of the LR. The GGS-MAPPIT was created to improve the flexibility of the system and to avoid interactions due to interaction with the LR. $\beta_c$-MAPPIT relies on functional complementation of STAT5 signalling and allows the analysis of protein interactions in haematopoietic cells.
Figure 14: Variants of MAPPIT

(A) LR-MAPPIT. The LR itself, devoid of its STAT3 recruiting tyrosine (Y1138) functions as bait protein. Upon stimulation, the two membrane proximal tyrosines can be phosphorylated by JAK2. Interaction of the prey protein with the LR, which may depend on phosphorylation, allows STAT3 recruitment and activation via gp130 and subsequent reporter induction. (B) β_c-variant of LR-MAPPIT. In β_c-MAPPIT a β_c receptor-based prey construct (inset) is used. The β_c receptor contains six tyrosine motifs in its cytoplasmatic tail of which three are known STAT5 recruitment sites. A prey in the β_c-MAPPIT method is fused to a part of the β_c receptor containing all three STAT5 recruitment sites. (C) GGS-MAPPIT. The bait protein is attached C-terminally to a variant of the chimeric EpoR-LR receptor. The cytosolic domain of the LR following the JAK2 recruitment site is replaced by 60 GGS triplets, preventing any background activation resulting from prey association with the LR. (D) β_c-variant of GGS-MAPPIT.
III. References


Eyckerman, S., and Tavernier, J. (2002). Methods to map protein interactions in mammalian cells: different tools to address different questions. Eur Cytokine Netw 13, 276-284.


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CHAPTER 5: Biological functions and signal transduction of leptin

The human body is evolutionary conditioned to endure periods of starvation. This is achieved principally by energy storage in adipose tissue, but also by reducing physical activity and lowering thermogenesis. The balanced maintenance of energy reserves throughout life is of vital importance. Therefore, a rigorous equilibrium between eating, physical activity and body metabolism exists and is strictly controlled by dedicated centres in the brain. The adipocyte-derived hormone leptin and its receptor emerged as key players in this control mechanism regulating the body weight. Moreover, there is a growing body of evidence that energy reserves, or the nutritional status, influence important physiological processes like the onset of puberty and reproduction, haematopoiesis and immune reactions.

I. Leptin and its receptor

Leptin refers to the Greek word leptos, which means ‘thin’. It was identified by positional cloning as the product of the obese (ob) gene (Zhang et al., 1994). This gene is truncated in the naturally occurring severely obese ob/ob mice. These mice display an early onset obese phenotype (figure 15) which is associated with a number of endocrinological disorders. Administration of recombinant leptin to ob/ob mice results in a reduced food intake, increased energy expenditure and weight loss, thereby supporting a role for leptin in the regulation of body weight (Halaas et al., 1995). Leptin is expressed as a 16kDa non-glycosylated protein with an intramolecular disulphide bond necessary for biological activity (Rock et al., 1996). Its structure was solved by crystallography and revealed a typical type I long chain cytokine structure (see chapter 1). The hormone is mainly secreted by white adipose tissue, and its circulating plasma levels correlate positively with body fat mass (Considine et al., 1996; Maffei et al., 1995). Lower levels of leptin production
could also be shown in other tissues, such as stomach, placenta and skeletal muscle (Bado et al., 1998; Masuzaki et al., 1997; Wang et al., 1998).

Figure 15: Phenotype of the \textit{ob/ob} mouse

A wild type mouse (right) and an obese \textit{ob/ob} mouse (left)

The leptin receptor (LR) was isolated as the product of the diabetes (\textit{db}) gene using an expression cloning strategy (Tartaglia et al., 1995). The gene is disrupted in the naturally occurring \textit{db/db} mice that have the same phenotype as \textit{ob/ob} mice. So far, six isoforms have been identified: one long form (LRlo or LRb), one soluble variant (LRe) and four short forms (LRsh or LRa, L Rc, L Rd and LRf) (figure 16). The different LR variants are generated through alternative splicing but a soluble LR can also be created by proteolytic ectodomain shedding of membrane-anchored LRs (Ge et al., 2002; Maamra et al., 2001). All isoforms possess an identical N-terminal extracellular domain but vary in their C-terminal intracellular part. The extracellular domain contains the typical extracellular characteristics of the class I cytokine receptor family. A rather unique feature is the presence of two extracellular CRH (CRH1 and 2) domains. These are separated by an Ig-like domain and followed by two FNIII domains (reviewed in (Zabeau et al., 2003)). The LRlo is the only isoform capable of efficient signalling. This LR variant is highly expressed within specific nuclei of the hypothalamus, known to be involved in body weight regulation. Expression could also be observed in different peripheral tissues (Fruhbeck, 2001; Lam et al., 2006; Sanchez-Margalet et al., 2002; Tian et al., 2002). The short
isoforms of the LR and especially the LRsh, are more abundantly expressed throughout the body (Fei et al., 1997). The short variants of the LR are proposed to be involved in the transport of leptin across the blood-brain barrier, leptin clearance or leptin signal transduction (Hileman et al., 2002; Murakami et al., 1997; Uotani et al., 1999). The secreted LRe is likely involved in the modulation of the plasma levels of circulating free leptin (Huang et al., 2001; Yang et al., 2004).

**Figure 16: Schematic representations of the murine LR isoforms**

The extracellular domain of all isoforms consists of a CRH domain, an Ig-like domain, a second CRH domain and two FNIII domains. The number of AA in each isoform is indicated. The box1 domain represents the proline rich region necessary for JAK binding.

II. Leptin: function and importance

**Body weight regulation**

Although daily food intake as well as energy expenditure can vary considerably, an individual's body weight remains remarkably constant over time. The physiological system that regulates body weight by balancing energy intake and expenditure is very strictly controlled by a short-term and long-term strategy. The short-term...
system mainly controls feeding via hunger and satiety signals. Satiety signals can be defined as rapidly released gastro-intestinal signals such as the hormone cholecystokinin that signals to the brain to stop an ongoing meal (Strader and Woods, 2005). Long-term regulation of energy balance is dependent on adiposity signal molecules that circulate at levels proportional to the body fat mass. Two cytokine-like hormones appear to be key players in this mechanism indicating sufficient long-term energy stores: leptin and insulin.

As circulating leptin levels correlate accurately with the body fat content, leptin is considered to function as an adipostat that communicates the status of body energy reserves to the brain (Friedman and Halaas, 1998; Maffei et al., 1995). Administration of leptin to rodents decreases food intake and increases energy expenditure (Campfield et al., 1996; Halaas et al., 1995). This demonstrates that leptin acts as an afferent satiety signal in a negative feedback mechanism that maintains the body weight at a constant level. Starvation leads to a decrease in fat

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Figure 17: Leptin functions as an adipostat, signalling the body energy stores to the brain
stores and concomitant drop in leptin levels, resulting in a reduction in energy expenditure and allows a longer survival. On the other hand, higher energy reserves will correlate with elevated leptin levels, reducing the food intake and augmenting the energy expenditure (figure 17). To enter the CNS, the adipocyte-derived leptin must pass the blood-brain-barrier (BBB), via a specific and saturable transport system that might engage the short isoform of the LR (Banks, 2004; Banks et al., 1996; Bjorbaek et al., 1998b). Once in the brain, leptin binds and activates its receptor expressed by neurons in certain nuclei of the hypothalamus. The importance of the brain and especially the hypothalamus as a direct target of the leptin is demonstrated by neural specific deletion of the LR which leads to obesity in mice (Cohen et al., 2001). LR activation will alter expression and release of neuropeptides, which then activate pathways that engage other brain regions, ultimately leading to a satiated feeling (Broberger, 2005; Friedman and Halaas, 1998). Leptin regulates two different populations of primary target neurons in the hypothalamus: anorexigenic and orexigenic neurons. Increased leptin levels will stimulate the anorexigenic (appetite-suppressive) neurons to express the satiety-related molecules cocaine-amphetamine-regulated transcript (CART) and pro-opion melanocortin (POMC), the precursor of the α-melanocyte-stimulating hormone (α-MSH). The orexigenic (appetite-stimulating) neurons are responsive to absence or low concentrations of leptin and express neuropeptide Y (NPY) and agouti-related protein (AgRP). The abundant NPY is a very potent orexigenic peptide that stimulates food consumption while AgRP antagonizes α-MSH action. Secondary target neurons process the NPY/AgRP and POMC/CART signals they receive. The orexigenic and anorexigenic systems act together and ultimately determine the response to peripheral signals (Friedman and Halaas, 1998).

A broader role for leptin

A growing body of evidence has qualified leptin as a pleiotropic molecule that is involved in a wide range of functions in the CNS and in the periphery. This is well illustrated by the phenotype of ob/ob and db/db mice: besides obesity, these mice display many defects in haematopoiesis, reproduction, angiogenesis, immune responsiveness, blood pressure control, bone formation and fetal development.
(Ducy et al., 2000; Fruhbeck, 1999; Harigaya et al., 1997; Holness et al., 1999; Lord et al., 1998; Sierra-Honigmann et al., 1998; Umemoto et al., 1997). The link between the energy status of the body and physiological processes might not be surprising, as adequate energy stores are crucial to support energy-demanding functions such as immunity and reproduction. Fasting or starvation leads to an attenuation of these processes, thereby prioritizing food collection and consumption. As mentioned before, expression of the LR1o was reported in many peripheral tissues and immune cells, including bone marrow, pancreatic β cells, endothelial cells, dendritic cells, NK cells and T lymphocytes (figure 18) (Fehmann et al., 1997; Gainsford et al., 1996; Lam et al., 2006; Lord et al., 1998; Sierra-Honigmann et al., 1998; Tian et al., 2002).

Figure 18: Localisation of functional LRs showing the involvement of leptin in peripheral effects

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Leptin has been increasingly recognized as a cytokine-like hormone with pleiotropic actions in modulating immune responses. Since cell-mediated immunity is an energy demanding process, leptin may provide an important link between the body’s energy status and the immune system. Leptin was characterized as a pro-inflammatory cytokine that functions as an early-phase reactant (Fantuzzi and Faggioni, 2000). It operates in both innate and adaptive immunity (La Cava and Matarese, 2004). In innate immunity leptin will for example affect functions of monocytes and macrophages including phagocytosis, release of pro-inflammatory cytokines and expression of adhesion molecules (Fantuzzi and Faggioni, 2000; Mancuso et al., 2002; Zarkesh-Esfahani et al., 2001). In adaptive immunity, leptin promotes the development of T cells by reducing apoptosis and it directs memory T cells towards a Th1 response (Howard et al., 1999; Lord et al., 1998). Given its role in regulating T cell-controlled immune responses, leptin may play a role in the onset of T cell-controlled autoimmune diseases. Indeed, studies on mouse models demonstrated an involvement of leptin in the pathogenesis of several autoimmune diseases including inflammatory bowel disease and multiple sclerosis (Matarese et al., 2007; Peelman et al., 2004). Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25(+) immunoregulatory T cells (Tregs) (Matarese et al., 2005). Tregs are known to suppress autoreactive responses mediated by CD4⁺CD25⁻ T cells and may influence the onset and progression of autoimmunity (Sakaguchi, 2004).

As a marker of the nutritional status, leptin has an essential role in reproduction. Leptin and its receptor have been found in reproductive tissues like ovary, oocytes, uterus and endometrium (Cervero et al., 2004; Kawamura et al., 2002; Ramos et al., 2005; Ryan et al., 2002). The hormone appears to be a permissive factor in the onset of puberty since leptin administration accelerates puberty in wild type mice and restores fertility in ob/ob mice (Chehab et al., 1996; Chehab et al., 1997; Gruaz et al., 1998). Leptin affects the hypothalamus-pituitary-gonadal axis, regulating gonadotrophin-releasing hormone and luteinising hormone secretion (Welt et al., 2004; Yu et al., 1997).
The involvement of leptin in haematopoiesis is suggested by the altered amount of blood cells seen in \textit{ob/ob} and \textit{db/db} mice (Bennett et al., 1996; Faderl et al., 2003). A direct role for leptin in haematopoiesis is proposed based on the expression of its receptor in fetal liver, bone marrow stromal cells and several haematopoietic cell lines (Cioffi et al., 1996; Konopleva et al., 1999). The capacity of the bone marrow cells to produce leptin provides further evidence for leptin in promoting the haematopoietic stem cells. This was further supported by colony formation studies where leptin stimulated the proliferation of stem cells and increased the numbers of lymphoid, erythroid and myeloid colonies (Bennett et al., 1996). Nevertheless, a direct role for leptin in the regulation of hematopoietic cells remains unclear.

**Leptin: clinical use and beyond**

The prevalence of overweight and obesity is escalating at an alarming rate, making it one of the most pressing health problems in the western world. In addition to the aesthetic considerations, obesity is indisputably linked with a number of serious health threats, including cardiovascular disease, stroke, type II diabetes and certain types of cancer (Calle et al., 1999; Kopelman, 2000). Although environmental and behavioural factors caused by economic development, urbanization and the media have been associated to the rise in global obesity, research demonstrated that obesity also has a significant genetic component. Given the remarkable weight loss of treated \textit{ob/ob} mice, leptin was initially expected to be a miracle drug for curing obesity. In strong contrast, trials in which recombinant leptin was administered to obese patients failed to live up to the expectations as only modest weight decrease was obtained (Heymsfield et al., 1999; Hukshorn et al., 2000). Only in a few cases of obesity, caused by absence of or aberrant leptin production (Montague et al., 1997; Ozata et al., 1999), administration of the hormone led to effective decrease in body weight (Farooqi et al., 1999). The majority of the obesity cases are associated with significantly elevated leptin levels, pointing to a failure to respond correctly to the leptin signal (Considine et al., 1996; El-Haschimi et al., 2000; Maffei et al., 1995). This so-called leptin resistance is discussed in text box 3. Leptin treatment was found to be effective for the treatment of hyperphagia caused by low leptin levels in humans with low body fat content (McDuffie et al., 2004; Welt et al., 2004).
Text box 3: Leptin resistance

Desensitization to leptin in obese individuals might result from defects in one of the three levels of leptin responses:

(i) Decreased transport across the blood-brain-barrier (BBB)

Impaired transport of leptin through the BBB has been demonstrated in rodents with diet-induced obesity (DIO), a model of obesity and leptin resistance in which rodents become obese by eating a high-fat diet. These DIO animals are resistant to peripheral leptin administration but lose weight when leptin was injected directly into the brain (El-Haschimi et al., 2000; Halaas et al., 1997).

(ii) Defects in LR activation and signal transduction

Impaired receptor expression results in a marked obese phenotype (e.g. db/db mice). In addition, aberrant signalling inhibition can cause central leptin insensitivity. SOCS3 and PTP1B are the two molecules that are most associated with attenuation of LR signalling and their enhanced activity can contribute to leptin resistance. Supportive of this, SOCS3 haploinsufficient or neural cell-specific deficient mice and PTP1B knock-out mice show hypersensitivity to leptin which protects them from high fat diet obesity (Cheng et al., 2002; Elchebly et al., 1999; Howard et al., 2004; Mori et al., 2004). However, neural PTP1B expression or activity is not altered by leptin or adiposity, suggesting that PTP1B may not underlie tempered leptin signalling in obesity. Conversely, SOCS3 expression increases in response to leptin and is elevated in the hypothalami of obese animals (Bjorbaek et al., 1998a; Munzberg et al., 2005; Tups et al., 2004). At present, most data confirm that alterations in cellular LR signalling, with a major role for SOCS3, have a major contribution in leptin resistance (El-Haschimi et al., 2000; Munzberg et al., 2005; Munzberg et al., 2004).

(iii) Impaired secondary leptin signalling

Defects in downstream effects of leptin in the neuronal circuit may also underlie leptin resistance. This comprises mutations in the genes encoding components of the neural circuit activated by leptin such as NPY, AgRP, POMC or CART. This is well illustrated by the obese phenotype observed for mice with loss-of-function mutations in the POMC encoding gene (Challis et al., 2004).
Accumulating evidence points to leptin as a potential link between obesity and cancer, especially in the development of breast, colorectal and prostate cancers (Garofalo and Surmacz, 2006). Leptin has been shown to act as a proliferative, mitogenic and pro-angiogenic agent promoting tumorigenesis of certain cancer cells (Bouloumie et al., 1998; Dieudonne et al., 2002; Horiguchi et al., 2006; Hu et al., 2002; Iversen et al., 2002; Sierra-Honigmann et al., 1998). The relevance of leptin signalling in cancer is reinforced by the fact that the LR is (over)expressed in several cancer cells (Garofalo et al., 2006; Hardwick et al., 2001; Ishikawa et al., 2004; Stattin et al., 2001). Until now, the association between circulating leptin levels and cancer risk has not been clear. Anyhow, recent studies suggest that for example breast carcinogenesis could also be induced by overabundance of locally produced leptin (Garofalo et al., 2006; Ishikawa et al., 2004).

In respect to its diverse functions in immunity, leptin has been explored as a potential target for therapeutic application in treating autoimmune diseases. Modulation of the hormone has been shown to target autoimmune disease in some mice models and further studies are on the way to test if antagonizing leptin activity can affect the pathogenesis of these immunological disorders in humans.

**III. Signalling via the leptin receptor**

The LR appears as pre-assembled dimers at the plasma membrane (Biener et al., 2005; Couturier and Jockers, 2003; White and Tartaglia, 1999). Leptin binding induces further clustering and conformational reorganisation of LR chains, thereby reorienting the intracellular domains in such a way that the associated JAKs become activated (Peelman et al., 2006; Zabeau et al., 2005; Zabeau et al., 2004). Three conserved tyrosine residues in the LR (Y985, Y1077 and Y1138, murine numbering) act as docking sites for downstream signalling molecules upon phosphorylation (figure 19) (Banks et al., 2000; Gong et al., 2007).
The JAK/STAT pathway

The LR can associate and activate both JAK1 and JAK2, but it is generally accepted that JAK2 is the main player under physiological conditions (Bjorbaek et al., 1997; Kloek et al., 2002; Muraoka et al., 2003). Interaction with JAK2 is mediated by the conserved box 1 motif, while the less conserved box 2, dispensable for JAK2 activation, likely functions in JAK2 selectivity (Bahrenberg et al., 2002; Kloek et al., 2002). Leptin stimulation was found to principally activate STAT3 in different in vivo studies (El-Hefnawy et al., 2000; Martin-Romero et al., 2000; McCowen et al., 1998).

Figure 19: Overview of the leptin signalling pathways

The Y1138 of the LR is embedded in an YXXQ motif and is responsible for the recruitment of STAT3 (Banks et al., 2000; Haan et al., 1999). Replacement of this residue by a serine in mice abolished STAT3 activation and as a result these knock-in mice became extremely obese (Bates et al., 2003), suggesting that STAT3 is a

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major intracellular mediator of leptin signalling. Nonetheless, these mice do not show the infertility and reduced size that is seen in db/db mice, indicative for the involvement of other signal transducers. Experiments with mutant LR constructs showed that deletion of the last tyrosine of the LR leads to a complete loss of STAT3 activation while STAT5 activity was still possible (Hekerman et al., 2005; White et al., 1997). Furthermore, leptin can stimulate proliferation of pancreatic β-cells and this effect may be mediated by activation of STAT5 (Islam et al., 2000). Convincingly, phosphorylation of STAT5 was recently demonstrated to regulate leptin signalling pathways in hypothalamic nuclei of mice (Gong et al., 2007). Phosphorylated Y1077 and Y1138 were identified as major docking sites for STAT5 (Gong et al., 2007; Hekerman et al., 2005).

The PI3K pathway

Leptin binding to its receptor also activates some components of the insulin signalling cascade including IRS1/2 through phosphorylated JAK2 (Elbatarny and Maurice, 2005). The IRS proteins bind to the regulatory unit p85 of PI3K to stimulate the catalytic domain. Recently, it was demonstrated that the association of the IRS4 adaptor to the P-Y1077 motif of the LR could also mediate PI3K recruitment (Wauman et al., 2007). Activated PI3K transforms PIP2 into PIP3, which stimulates PDK1 for the phosphorylation and activation of Akt. This will ultimately result in the induction of cAMP phosphodiesterase PDE3B and a reduction of cAMP levels. In the hypothalamus, regulation of cAMP has been shown to play a critical role in feeding and body weight, making the PI3K pathway an important component of leptin signalling in energy homeostasis (Gillard et al., 1998; Minokoshi et al., 2004; Shimizu-Albergine et al., 2001).

The MAPK pathway

Tyrosine 985 of the LR plays an important role in leptin-induced MAPK activation by acting as a docking site for the protein tyrosine phosphatase, SHP-2 (Li and Friedman, 1999). Since SHP-2 can also negatively regulate leptin-induced
JAK/STAT signalling, it appears that this phosphatase can have opposite functions (Carpenter et al., 1998; Li and Friedman, 1999; Zhang et al., 2004). SHP-2 is phosphorylated by JAK2 and forms a docking site for the adaptor protein Grb2 leading to activation of the ERK signalling cascade (Banks et al., 2000). Alternatively, ERK can also be activated by direct binding of SHP-2 to JAK2 (Bjorbaek et al., 2001). Leptin-triggered activation of MAPK was observed both centrally and peripherally. Regulation of calcium influxes involving MAPK activity was shown in hypothalamic neurons upon leptin stimulation (Jo et al., 2005). In peripheral tissues, leptin influences adipogenesis in preadipocytes and induces production of nitric oxide (NO) in white adipocytes via MAPK activation (Machinal-Quelin et al., 2002; Mehebik et al., 2005). Furthermore, leptin-induced MAPK is involved in full activation of the DNA binding of STAT3 by mediating serine phosphorylation at position S727 of STAT3 (O'Rourke and Shepherd, 2002).

IV. Negative regulation of leptin receptor signalling

LR signal modulation is reviewed in chapter 8.
V. References


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Chapter 5: Biological functions and signal transduction of leptin


Chapter 5: Biological functions and signal transduction of leptin
**Scope of the thesis**

Since the discovery of the SOCS family only a decade ago, important advances have been made in understanding the mode of action by which these regulators attenuate cytokine signalling. However, unresolved issues include the involvement of SOCS in the modulation of specific pathways and the mechanisms controlling stability and regulation of these proteins. The importance of this is underscored by the contribution of inappropriately regulated SOCS activity to several pathologies including growth related diseases, cancer and inflammatory diseases. In this thesis, we aimed at further elucidating the mechanisms underlying SOCS regulation, thereby making extensive use of the mammalian two-hybrid MAPPIT technique. MAPPIT allows analysis of protein-protein interactions in a physiological context and is well suited for examining signal transduction pathways since it is capable of identifying interactions that are transient and weak or that depend on tyrosine phosphorylation. Throughout this thesis, several adaptations of the MAPPIT strategy were developed and applied in order to increase the sensitivity of the system and to expand the technology to different cellular backgrounds.

The pivotal role of leptin in body weight regulation is demonstrated by the extreme obese phenotypes observed in mice containing defects in either leptin or its receptor. The importance of a strict control of leptin activity is underscored by the state of leptin resistance commonly found in obesity which may be caused by aberrant attenuation of hypothalamic leptin signalling. A first part of this work focuses on the role of SOCS proteins in the modulation of LR signal transduction. SOCS3 is considered as a major inhibitor of leptin signalling and is proposed to be involved in the development of this leptin resistance. Still, expression of other SOCS members is induced upon leptin stimulation. A first objective was to define if other SOCS proteins are recruited to the activated LR and thereby contribute to modulation of leptin responses. Using MAPPIT and peptide affinity chromatography we studied interactions between the LR and SOCS in greater detail and identified two novel interactions of SOCS with phosphorylated tyrosine motifs. We further
analysed the differential binding mode of these interaction partners and the functionality of the interactions (chapter 6).

Beside its effect on hypothalamic body weight regulation, leptin is also involved in a broad range of peripheral functions including immunity, haematopoiesis and reproduction and may also contribute to the development of disorders like autoimmune diseases. The role of leptin in these peripheral effects is far from clear. Since different inconsistencies concerning leptin signalling in haematopoietic cells remain, we investigated signalling events mediated by the LR using a novel MAPPIT variant, βc-MAPPIT, which allows analysis in a haematopoietic background. This way, we characterised several interactions (including SOCS proteins) with the LR that are likely relevant for haematopoietic leptin signalling (chapter 7).

The essential role of the SOCS box was demonstrated by the defective phenotypes of transgenic mice expressing SOCS box deletion mutants of SOCS1 and SOCS3 (Boyle et al., 2007; Zhang et al., 2001). Via Elongin B/C recruitment, the SOCS box links associated molecules to E3 ligase activity and subsequent proteasomal degradation (Kamura et al., 1998; Zhang et al., 1999). The SOCS box can also function as an adaptor, coupling SOCS actions to other downstream signalling pathways including the MAPK pathway. Furthermore, this domain was found to be involved in the regulation of SOCS protein levels. The second part of this thesis deals with the versatile effects of the SOCS box on SOCS functions and cytokine signalling. Increasing evidence makes clear that SOCS proteins not only act as inhibitors of cytokine responses but exert broader regulatory mechanisms. In particular for SOCS2, data indicate that it can have both inhibitory and stimulatory effects on cytokine responses. This dual effect led to the speculation that SOCS2 interferes with the inhibition of other SOCS proteins. We investigated this hypothesis in the context of different cytokine pathways by using functional assays. MAPPIT and co-immunoprecipitation experiments were performed to study the interactions between SOCS2 and other SOCS proteins. The contribution of the SOCS box domain and the involvement of proteasomal degradation in this SOCS cross-modulation were also examined. Finally, we tested whether, in analogy to SOCS2, other SOCS members exerted comparable interfering characteristics (chapter 9).

As previous studies reported that the SOCS box of CIS is essential for interaction with receptor motifs (Lavens et al., 2007), we aimed to clarify the role of the SOCS
box in substrate interaction of SOCS proteins. More specifically, we evaluated the involvement of Elongin B/C recruitment on substrate binding and functionality of CIS. We pursued by testing if this SOCS box-dependency is unique for CIS. Based on models of CIS, a structural basis was provided for this regulatory mechanism controlling SH2 domain function (chapter 10).

Taken together, the SOCS box domain emerges as a versatile regulatory module controlling SOCS activity and cytokine signal transduction pathways at multiple levels (reviewed in chapter 11).

References


Part II: The role of SOCS proteins in regulation of LR signalling
CHAPTER 6: Interaction pattern of CIS and SOCS2 with the leptin receptor

I. Introduction

SOCS3 is considered to be one of the main players in the attenuation of leptin signalling and is suggested to underlie the development of leptin resistance commonly found in obesity (Bjorbaek et al., 1999; Bjorbaek et al., 1998). Yet, expression of other SOCS members, including SOCS1, SOCS2 and CIS, is also induced in response to leptin (Emilsson et al., 1999; Lavens et al., 2006; Motta et al., 2004). Within the context of this PhD project, the MAPPIT data set demonstrating the interaction of CIS and SOCS2 with the LR was generated and the interactions were confirmed with biochemical methods. Surprisingly, the closely related proteins CIS and SOCS2 display differential binding capacities: both molecules bind to phospho (P)-Y1077, but only CIS appears to interact with the P-Y985 of the LR. CIS and SOCS2 are believed to mainly inhibit STAT5 signalling. Recently, phosphorylated Y1077 of the LR was revealed as a major docking site for STAT5 (Gong et al., 2007). We report here that SOCS2 can block the binding of the SH2 domain of STAT5a at this tyrosine. Surprisingly, we observed that SOCS2 interferes with CIS association at P-Y1077 but also at the Y985 position, although SOCS2 itself does not interact with this tyrosine. We further examined this observation and found that SOCS2 could interact with CIS and that the interfering effect depends on Elongin B/C recruitment to SOCS2. This suggests that proteasomal degradation of CIS might be involved in this inhibitory mechanism. Besides the well-established inhibitory effect of SOCS3 on hypothalamic leptin signalling, other SOCS proteins bind the LR and most probably are involved in the modulation of leptin signalling in other cell types.
II. Article: A complex interaction pattern of CIS and SOCS2 with the leptin receptor.


III. References


A complex interaction pattern of CIS and SOCS2 with the leptin receptor

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Summary

Hypothalamic leptin receptor signalling plays a central role in weight regulation by controlling fat storage and energy expenditure. In addition, leptin also has direct effects on peripheral cell types involved in regulation of diverse body functions including immune response, bone formation and reproduction. Previous studies have demonstrated the important role of SOCS3 (suppressor of cytokine signalling 3) in leptin physiology. Here, we show that CIS (cytokine-inducible SH2 protein) and SOCS2 can also interact with the leptin receptor. Using MAPPIT (mammalian protein-protein interaction trap), a cytokine receptor-based two-hybrid method operating in intact cells, we show specific binding of CIS with the conserved Y985 and Y1077 motifs in the cytosolic domain of the leptin receptor. SOCS2 only interacts with the Y1077 motif, but with higher binding affinity and can interfere with CIS and STAT5a prey recruitment at this site. Furthermore, although SOCS2 does not associate with Y985 of the leptin receptor, we find that SOCS2 can block interaction of CIS with this position. This unexpected interference can be explained by the direct binding of SOCS2 on the CIS SOCS box, whereby elongin B/C recruitment is crucial to suppress CIS activity.

Key words: Leptin receptor, SOCS proteins, Signalling, Cross-regulation

Introduction

Leptin plays a major role in the regulation of energy homeostasis and food intake. Produced mainly in white adipose tissue (Zhang et al., 1994), it translocates through the blood-brain-barrier to target the leptin receptor (LR) in the hypothalamus. Although six LR splice variants can exist, the LR isoform with an extended cytoplasmic domain (LR1o) is the predominant signalling variant (Ghilardi et al., 1996). A short variant (LRsh) is abundantly expressed in the choroid plexus, brain microvessels, lung and kidney and may participate in leptin transport across the blood-brain barrier (Bjorbaek et al., 1998b; Boado et al., 1998). Next to its effect in weight regulation, leptin is also involved in a broad range of other functions including reproduction, bone formation, growth, immune regulation, angiogenesis and glucose and insulin metabolism.

The LR was addressed to the type I cytokine receptor family based on sequence homology (Tartaglia et al., 1995). It is closely related to the gp130 receptor family, especially gp130, oncostatin M (OSM) and leukaemia inhibitory factor (LIF) receptors, and to the G-CSF receptor (granulocyte-colony stimulating factor) (Zabeau et al., 2003). Leptin typically signals through the JAK-STAT pathway. An overview of LRsignalling events is shown in Fig. 1A. The LR carries three conserved tyrosines in its cytoplasmic tail (positions Y985, Y1077 and Y1138 in the murine LR), whereby the membrane distal tyrosine Y1138 is embedded in a STAT3 (signal transducer and activator of transcription) recruitment motif. The activated receptor recruits STAT3 molecules through their SH2 domain (Baumann et al., 1996; Vaisse et al., 1996), and, after tyrosine phosphorylation, they translocate as homodimers to the nucleus to induce specific gene expression.

Knock-in mice containing a Y1138S mutation reveal a severe obese phenotype but do not show the infertility and reduced size that occurs in db/db mice (Bates et al., 2003). This observation, together with the wide range of leptin-responsive cell types, suggests that alternative signalling pathways must exist. Leptin-dependent activation of STAT1 and STAT5 was demonstrated in vitro (Baumann et al., 1996; Hekerman et al., 2005). In addition, recruitment of SH2-containing phosphatase SHP-2 to the phosphorylated Y985 position is responsible for leptin-induced MAPK signalling, although an additional pathway for activation of this signalling cascade directly by JAK2 has been suggested (Bjorbaek et al., 2001). Leptin also induces phosphorylation of IRS-1 and IRS-2 (Duan et al., 2004) and activates phosphatidylinositol 3-kinase (PI-3K), as demonstrated in several cell lines (Cohen et al., 1996; Kim et al., 2000). A role for JAK2 in activation of the PI-3K pathway through the JAK2-interacting protein SH2-B and recruitment of IRS-1 or IRS-2 was also reported (Duan et al., 2004). SH2-B and leptin-activated hypothalamic PI-3K both appear essential for weight regulation (Niswender et al., 2001; Ren et al., 2005). Recently, an inhibitory effect of leptin on hypothalamic AMPK (AMP-activated protein kinase) activity was reported. AMPK is proposed to act as a ‘fuel gauge’ to an intracellular energy sensor cascade and
its activation in the hypothalamus promotes food intake (Minokoshi et al., 2004).

CIS (cytokine-inducible SH2 protein) was the founding member of the SOCS (suppressor of cytokine signalling) family, now consisting of eight proteins: SOCS1-7 and CIS. SOCS proteins typically have an SH2-domain, an N-terminal

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**Fig. 1.** (A) Overview of LR signalling and its interaction partners. The murine LR carries three conserved tyrosines in its cytoplasmic tail at positions Y985, Y1077 and Y1138. JAK2 is constitutively associated with the LR at the conserved Box 1 and 2 motifs. Upon leptin stimulation, the JAKs become fully activated through cross-phosphorylation and phosphorylate the tyrosine residues in the receptor. STAT3 is recruited to the phosphorylated Y1138 docking site. Upon phosphorylation, STAT3 translocates as dimers to the nucleus, and induces specific gene expression. SHP2 is recruited to the Y985 docking site and couples to the Ras/Raf signalling cascade. The PI-3K pathway is also involved in LR signalling. Tyrosines Y985 and Y1077 take part in negative regulation of the leptin signal by binding SOCS3. PTP-1B is involved in negative regulation by dephosphorylation of JAK2 after internalisation of the LR complex. (B) MAPPIT principle. A particular bait protein is linked C-terminally to the chimeric receptor consisting of the extracellular part of the EpoR and the intracellular part of the LR with all three tyrosines mutated to phenylalanine, whereas the prey protein is fused to the STAT3 recruitment sites of the gp130 chain. The bait-receptor is incapable of recruiting STAT3 upon stimulation. However, when bait and prey proteins interact, the C-terminal part of the gp130 chain is brought in close proximity to the JAK kinases allowing its tyrosine phosphorylation and subsequent STAT3 activation. Read-out is based on a STAT3-responsive reporter construct. (C) GGS-MAPPIT. For GGS-MAPPIT the bait protein is attached C-terminally to a variant of the chimeric EpoR-LR receptor. The cytosolic domain of the LR following the JAK2 association domain is replaced by a GGS-array, preventing any background activation resulting from prey association with the LR-F3. (D) LR-MAPPIT. Here, the LR itself functions as bait protein. Owing to the Y1138F mutation, no STAT3 recruitment or activation can occur. Upon stimulation, the two membrane proximal tyrosines can nevertheless be phosphorylated by JAK2. Interaction of the prey protein with the LR, which may depend on phosphorylation, allows STAT3 activation and subsequent reporter induction.
preSH2-domain and a C-terminal SOCS box (Starr et al., 1997). The SOCS box targets signalling proteins to the proteasome for degradation by recruitment of an ubiquitin-transferase system (Kamura et al., 1998). SOCS1 and 3 also carry a KIR (kinase inhibitory region) domain that may act as a pseudosubstrate for direct inhibition of JAK kinase activity. Although SOCS1 associates with JAK2, SOCS3 binds the receptor in close proximity to the kinase and shows only weak affinity for JAK2 (Kubo et al., 2003). Competition for binding to shared recruitment sites can also contribute to the negative regulation of signalling pathways, as exemplified for CIS and SOCS2 in case of STAT5 recruitment at the growth hormone receptor (Greenhalgh et al., 2002a; Ram and Waxman, 1999).

SOCS3 was identified as a potent inhibitor of LR signalling. It associates predominantly with the pY985 motif in the LR. Weak interaction at position pY1077 may explain its additive effect on inhibition of LR signalling (Bjorbaek et al., 2000; Eyckerman et al., 2000). SOCS3 is rapidly expressed in the hypothalamic upon leptin stimulation making it part of a STAT3-mediated negative feedback system (Bjorbaek et al., 1998a; Dunn et al., 2005). Recently, PTP-1B was also identified as a negative mediator of LR signalling, targeting both the JAK-STAT and the MAPK pathway (Kaszubsksa et al., 2002).

It is well established that in many cytokine receptor systems multiple SOCS proteins can be involved in regulation. In the case of the growth hormone, erythropoietin and prolactin receptors, this includes CIS, SOCS2 and SOCS3. Since leptin can activate STAT5 (Baumann et al., 1996; Hekerman et al., 2005) and since CIS and SOCS2 are known regulators of STAT5 recruitment (Ram et al., 1999; Greenhalgh et al., 2002a), we questioned whether CIS or SOCS2 could be involved in LR signalling. Consistent with this, highly conserved tyrosine-based motifs compatible with CIS and SOCS2 association are present in the LR. Also, leptin can induce CIS and SOCS3 expression, and to a lesser extent SOCS2 in insulinoma cells (data not shown). To analyse these interactions with the LR we used two alternative versions of the MAPPIT (mammalian protein-protein interaction trap) strategy (Fig. 1). We observed differential binding of CIS and SOCS2 with the LR and demonstrate two distinct mechanisms for functional interference by SOCS2.

Results
Cytokine receptor signalling and design of MAPPIT experiments
An overview of signalling through the leptin receptor (LR) is shown in Fig. 1A, and is described in more detail in the introductory section. With MAPPIT we developed a new method to analyse protein interactions in mammalian cells (Eyckerman et al., 2001). MAPPIT bait constructs were originally designed as chimeric receptors, consisting of the extracellular part of the erythropoietin receptor (EpoR) fused to the transmembrane and intracellular regions of a STAT3 recruitment-deficient LR, with a C-terminally attached bait. MAPPIT prey constructs are composed of a prey polypeptide fused to a part of the gp130 chain carrying 4 STAT3 recruitment sites. Co-expression of interacting bait and prey leads to functional complementation of STAT3 activity that can be measured with the STAT3-responsive rat pancreatitis-associated protein 1 (rPAPI) promoter-luciferase reporter (Fig. 1B). Intrinsically to this strategy, both modification-independent and tyrosine phosphorylation-dependent interactions can be detected.

To monitor interactions with isolated tyrosine motifs of the LR, we developed a MAPPIT configuration whereby the cytosolic domain of the LR is replaced by a large array of Gly-Gly-Ser (GGS) repeats (Fig. 1C). The MAPPIT technique also allows the analysis of interactions with the LR itself by simple mutation of the Y1138 STAT3-recruitment motif to phenylalanine (Fig. 1D). LRs with different combinations of Y to F mutations of the two other conserved tyrosine motifs (located at positions Y985 and Y1077) were used. This allows the study of protein associations with the LR in its normal oligomeric configuration.

MAPPIT analysis of CIS and SOCS2 interactions with the LR
To determine interaction with the LR, the CISprey fusion protein was transiently co-expressed with the LR(YYF) mutant and the luciferase reporter construct (Fig. 2A). Clear induction of luciferase activity indicated that CIS interacts with the LR. MAPPIT experiments using LR(YFF), LR(FYF) or LR(F3) showed that CIS can interact with both Y985 and Y1077 motifs, whereas no interaction was detected with the LR lacking tyrosines. In a similar way we also tested the SOCS2-LR interaction (Fig. 2A). SOCS2 clearly associates with the LR, but only at position Y1077. Expression of the LR mutants was analysed using a leptin-SEAP binding assay (Fig. 2B), and expression of the FLAG-tagged CIS and SOCS2 preys was revealed by immunoblotting using an anti-FLAG antibody (Fig. 2C).

Phosphopeptide binding analysis
We confirmed the specific interaction of SOCS2 with pY1077 of the LR using a biochemical strategy (Fig. 3). FLAG-tagged SOCS2 or CIS proteins were expressed in HEK293T cells and total cell lysates were incubated with the biotinylated peptides encompassing the LR phosphorylated or non-phosphorylated Y1077 or Y985 motifs to verify (phospho)tyrosine-specific association. SOCS2 clearly interacted with the phosphorylated Y1077 motif but not with the phosphorylated Y985 motif, confirming its specific phosphorylation-dependent interaction with the LR at position Y1077. Association of CIS was found with neither pY985 nor pY1077 indicating that these interactions may be to weak or short-lived to be detected by phosphopeptide affinity chromatography (data not shown).

Relative binding affinities of the CIS and SOCS2 interactions with the LR
To gain further insight into their relative binding affinities for the LR, the CISprey or SOCS2prey were co-expressed with wild-type CIS. Although CIS expression markedly reduced the CISprey signal through both LR(YFF) and LR(FYF), it did not lead to any inhibition of the SOCS2prey signal through Y1077. Conversely, co-expression of wild-type SOCS2 with the CISprey protein clearly diminished the MAPPIT signal at the Y1077 position in the LR whereas the SOCS2prey signal is only partially reduced (Fig. 4A). These results confirm that CIS interactions with Y985 and Y1077 of the LR are weak or
transient, whereas the association of SOCS2 with Y1077 is more stable and therefore not easy to compete. It is quite surprising that SOCS2 can inhibit the MAPPIT signal of the CISprey protein through Y985 since SOCS2 is not interacting with this position. Expression levels of the FLAG-tagged proteins were confirmed by immunoblotting using an anti-FLAG antibody (Fig. 4B).

Analysis of CIS and SOCS2 interactions with the LR using GGS-MAPPIT

A new adaptation of the classic MAPPIT method, called GGS-MAPPIT (Fig. 1C), was used to confirm the interaction of CIS and SOCS2 with the LR. In this configuration the cytosolic domain of the LR, following the JAK2 interaction site, is replaced by 60 GGS repeats. GGS triplet repeats are often used as hinge sequences for their known structural flexibility. By using this GGS-MAPPIT strategy any background prey association with the LR-F3 is prevented. The bait constructs containing the LR motifs surrounding Y985 or Y1077 were transiently co-transfected with the prey construct and the rPAP

Fig. 3. SOCS2 interaction with the peptide matching the Y1077 motif of the LR is phosphorylation dependent. FLAG-tagged SOCS2 was expressed in HEK293T cells and lysates were incubated with phosphorylated or non-phosphorylated peptides corresponding to the Y1077 or Y985 motif. Immunoblotting with anti-FLAG antibody revealed specific interaction of SOCS2 with the tyrosine-phosphorylated Y1077 motif.

Fig. 4. Stability of the CIS and SOCS2 interactions with the LR. (A) HEK293T cells were transiently co-transfected with plasmids encoding different pMET7-LR variants, the pMG2-CIS or pMG2-SOCS2 prey construct, pEF-FLAG-I/mCIS or pEF-FLAG-I/mSOCS2, or the appropriate amount of mock vector together with the pXP2d2-rPAP1-luci. The transfected cells were either stimulated for 24 hours with leptin or were left untreated (NS, not stimulated). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (leptin stimulated/NS) + s.d. (B) Western blot analysis of CISprey, SOCS2prey, CIS and SOCS2 expression. Expression of the FLAG-tagged fusion proteins, CIS and SOCS2 was verified on lysates of transfected cells using anti-FLAG antibody.
luciferase reporter construct in HEK293T cells. Using GGS MAPPIT we were again able to detect the interaction of CIS with both the Y985 and Y1077 motifs, whereas SOCS2 only interacts with the Y1077 motif, but not with the pY985 motif (Fig. 5A). We tested this GGS-MAPPIT strategy further in erythroleukaemic TF-1 cells and obtained similar results as those found in HEK293T cells (Fig. 5B). A full-length FKBP12 bait was used to evaluate non-specific binding of the CIS and SOCS2 preys. FACS analysis, using antibodies against the extracellular domain of the EpoR, allowed monitoring of the expression of the different GGS baits (Fig. 5C).

**SOCS2 interferes with STAT5a recruitment**

We previously showed that STAT5 can be activated by the LR upon recruitment to the LR Y1077 and Y1138 motifs (Hekerman et al., 2005). Given the strong interaction of SOCS2 at position Y1077 we examined whether SOCS2 can interfere with STAT5 association at this position. The SH2 domain of STAT5a was inserted in a prey construct and used in MAPPIT experiments using the Y1077 motif as bait in GGS-MAPPIT. In HEK293T cells, co-expression of SOCS2 or a SOCS2 mutant lacking the entire SOCS box completely abolished the MAPPIT signal. Similarly, co-expression of SOCS2 Δbox in TF-1 cells also abrogated the MAPPIT signal, thus excluding a role for elongin B/C recruitment in this suppressive effect (Fig. 6A,B). Similar data were obtained using the LR(FYF) as bait (data not shown). We conclude that SOCS2 can compete with STAT5a association at the pY1077 motif.

**SOCS2 interacts with the SOCS box of CIS**

Given the discrepancy between binding experiments at position Y985, i.e. SOCS2 interferes with CIS binding without...
interacting with this recruitment motif itself, we examined whether SOCS2 can directly associate with CIS. We first used a MAPPIT configuration with CIS as bait. Here, SOCS2 clearly interacted specifically with full-length CIS (Fig. 7A). In Fig. 7B, we confirmed this interaction by co-immunoprecipitation. Next we looked at association of SOCS2 with the SOCS box of CIS in a MAPPIT experiment and also observed clear interaction (Fig. 7C).

Elongin B/C recruitment is involved in SOCS2 interference with receptor-binding of CIS

We developed a mutant of SOCS2, SOCS2(LC-QQ), in which elongin B/C recruitment is abrogated by mutating leucine 163 and cysteine 167, analogous to an elongin B/C recruitment-deficient SOCS1 mutant reported before (Kamura et al., 1998). We mutated both residues to glutamines to minimise structural alterations. Elongin B/C association was analysed using a two-step purification method, TAP2, based on the classic TAP method (Puig et al., 2001). This sequential purification procedure involves a first protein A tag-based step, followed by TEV protease cleavage to remove the protein A part of the tag and followed by a FLAG-tag-based immunoprecipitation step. Clearly, this SOCS2(LC-QQ) mutant no longer interacted with elongin B or C (Fig. 8A). Furthermore, this SOCS2 mutant as a prey protein still bound CIS in a MAPPIT experiment (Fig. 8B).

We next examined the interference of SOCS2 with CIS binding in more detail. At position Y985, the inhibitory effect by co-expression of SOCS2 was completely lost when using the SOCS2(LC-QQ) mutant. Recruitment of elongin B/C to the SOCS box of SOCS2 thus appeared essential for interference with CIS interaction at this position. By contrast, no difference was observed for the SOCS2(LC-QQ) mutant at the Y1077 position, clearly in line with a direct competition with CIS binding at this site (Fig. 8C).

Discussion

MAPPIT allows the study of protein-protein interactions in the physiologically highly relevant context of intact human cells. Here we used several variations of the MAPPIT concept to study the interactions of two members of the SOCS protein family, CIS and SOCS2, with the murine LR long isoform. CIS and SOCS2 preys were shown to interact with specific tyrosine motifs, either within the full LR configuration or as isolated baits. Interactions were demonstrated in two different cell types: epithelial HEK293T cells as well as the haemopoietic TF-1 cell line.

CIS binding was observed with the conserved mLR Y985 and Y1077 tyrosine-based motifs. By contrast, SOCS2 interacted only at the Y1077 position. In all cases, a Y to F mutation abrogated signalling, indicative of the phosphorylation-dependent nature of the interactions. We compared MAPPIT-based interaction analysis with a biochemical approach using...
Fig. 8. (A) Generation of a SOCS2 mutant deficient in elongin B/C binding. HEK293T cells were transiently transfected with the pMET7TAP2-SOCS2 and pMET7TAP2-SOCS2(LC-QQ) constructs. Cell lysates were purified using the TAP2 tag and loaded on a polyacrylamide gel and silverstained. From a parallel experiment, the indicated bands were identified as cullin 5, elongin B and elongin C by mass spectrometry. (B) The SOCS2(LC-QQ) mutant still binds CIS. HEK293T cells were transiently co-transfected with plasmids encoding the chimeric EpoR-LR(F3) construct as a negative control or with the CIS SOCS box bait, and the pMG2-SOCS2 or pMG2-SOCS2 (LC-QQ) prey constructs, combined with the pXP2d2-rPAP1-luci. The transfected cells were either stimulated for 24 hours with Epo or were left untreated (NS, not stimulated). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (Epo stimulated/NS) + s.d. (C) Differential effects of the SOCS2(LC-QQ) mutant on CIS interaction with the LR recruitment motifs. HEK293T cells were transiently co-transfected with plasmids encoding different pMet7-LR variants, the pMG2-CIS prey construct, pMet7-FLAG-SOCS2 or pMet7-FLAG-SOCS2(LC-QQ), or the appropriate amount of mock vector together with the pXP2d2-rPAP1-luci. The transfected cells were either stimulated for 24 hours with leptin or were left untreated (NS, not stimulated). Luciferase measurements were performed in triplicate. Data are expressed as mean fold induction (leptin stimulated/NS) + s.d.
affinity chromatography with phosphorylated and non-phosphorylated peptides matching the Y1077 or the Y985 motifs. The interaction between SOCS2 and the pY1077 motif was readily demonstrated, in contrast to CIS and its matching phosphopeptides. This is probably due to the more transient or weak nature of the latter interactions. In line with this proposal, competition experiments showed that whereas CIS overexpression could clearly interfere with CIS-prey binding to either motif, no cross-competition with the SOCS2-prey occurred. Conversely, SOCS2 could easily interfere with CIS-prey binding to pY1077.

Previous reports indicated that the tyrosine at position Y1077 of the receptor was not phosphorylated and was not involved in LR signalling (Banks et al., 2000; Li and Friedman, 1999). However, several observations contradict this supposition. We reported earlier that SOCS3 can interact with the Y1077 domain, although in a rather weak manner, and that this interaction was dependent on tyrosine phosphorylation (Eyckerman et al., 2000). More recently, Y1077 was also reported to induce STAT5 activation (Hekerman et al., 2005). Consistent with a functional role, Y1077 is present in a highly conserved motif, with great similarity to the conserved Y985 domain (Eyckerman et al., 2000). Our findings now lend further support for the important role of the pY1077 motif in LR signalling with two more members of the SOCS protein family interacting at this position, whereby SOCS2 can interfere with CIS and STAT5a prey recruitment.

Very surprisingly, SOCS2 not only interfered with CIS-prey interaction at position Y1077, but also at the Y985 motif without binding this site itself. We provided an explanation for this unexpected finding by showing that SOCS2 directly binds to the SOCS box of CIS. Abrogation of the elongin B/C recruitment ability of SOCS2 had no influence on its association with CIS, but its ability to eliminate CIS receptor binding at position Y985 was completely lost, implying that ubiquitylation and proteasomal degradation of CIS is involved. Very recently, it was reported that SOCS2 also interferes with SOCS3-dependent inhibition of IL-2 and IL-3 signalling (Tannahill et al., 2005). Together, these findings point to an additional, new level of SOCS-mediated signalling control. Reminiscent of this, both mice lacking SOCS2 and SOCS2 transgenic mice exhibit increased growth due to prolonged growth-hormone-dependent STAT5 activity (Greenhalgh et al., 2002b; Metcalf et al., 2000). This dual effect of SOCS2 was also observed in vitro because low SOCS2 doses moderately inhibit GH signalling whereas higher levels positively regulate signalling, probably through interference with SOCS1 function (Favre et al., 1999; Greenhalgh et al., 2005). Our interaction analysis clearly implicates a complex biological role for SOCS2 and suggests an explanation for the abovementioned duality: SOCS2 can interfere with cytokine signalling through direct interaction with receptors, but can also enhance signalling by eliminating other SOCS proteins through proteasomal degradation. This latter effect may reflect a crucial physiological role of SOCS2 in restoring cellular responsiveness after cytokine activation. In line with this, SOCS2 is usually induced at later time points compared with CIS, SOCS1 and SOCS3 (Adams et al., 1998; Pezet et al., 1999; Tannahill et al., 2005). Detailed quantitative analyses will be required to understand this balancing act in full.

Leptin resistance, which occurs in a majority of obese individuals, may be situated at different levels, e.g. saturation of leptin transport through the blood-brain barrier or aberrations in LR signalling in hypothalamic neurons (El-Haschimi et al., 2000). LR Y1138S knock-in mice are severely obese and fail to activate STAT3, implying a dominant role for STAT3 in leptin-mediated regulation of the energy balance (Bates et al., 2003). Aberrant negative feedback control of LR signalling may contribute to leptin resistance and obesity, because augmented leptin sensitivity and resistance to diet-induced obesity was observed in neural-cell-specific SOCS3 conditional-knockout mice or in SOCS3-haploinsufficient mice (Howard et al., 2004; Mori et al., 2004). In contrast to SOCS3, a negative regulatory role for CIS and SOCS2 on the hypothalamic LR STAT3 pathway is questionable. Bjorbaek and colleagues reported that JAK2 phosphorylation is inhibited by SOCS3 upon leptin stimulation in COS cells but not by SOCS2 or CIS, which both lack a KIR domain at the N-terminus (Bjorbaek et al., 1999). Similarly, we did not observe any clear inhibitory effect on LR signalling through STAT3 by either CIS or SOCS2 (data not shown). This is not unexpected because STAT3 recruitment occurs at the Y1138 motif. Since expression of SOCS2 or CIS is also not up-regulated in the hypothalamus upon leptin administration in mice, a role in LR STAT3 signalling is thus unlikely (Bjorbaek et al., 1998a).

CIS and SOCS2 can function through competition with STAT binding at the receptor recruitment site. This mechanism, for example, underlies down-regulation of STAT5 activation by both CIS and SOCS2 upon growth hormone receptor (GHR) activation (Greenhalgh et al., 2002a; Ram and Waxman, 1999). Likewise, the physiological role for CIS and SOCS2 on LR signalling through the Y985 and Y1077 motifs may involve inhibition of recruitment of downstream signalling moieties. This may be particularly relevant in peripheral cell types, known to respond to leptin. Experiments on MLR (mixed-lymphocyte reaction), resulting from the culture of T cells with major histocompatibility complex (MHC)-incompatible stimulator cells, indicated that leptin promotes proliferation of CD4+ T cells (helper T cells, Th) and induces a shift in activation of Th1 cells, associated with elevated secretion of pro-inflammatory cytokines including interleukin-2 (IL-2) and interferon-γ (IFN-γ) (Lord et al., 1998). Intriguingly, CIS transgenic mice show altered helper T-cell development with a switch toward Th2 cell response, accompanied by increased IL-4 levels (Matsumoto et al., 1999). CIS may thus be involved in the leptin-dependent modulation of the Th1/Th2 balance. SOCS2 knock-out mice showed a remarkable increase in size whereas growth retardation was observed in CIS transgenic mice. Both SOCS proteins were identified as negative regulators of GHR signalling, presumably through STAT5 (Matsumoto et al., 1999; Metcalf et al., 2000). Considering the decreased linear growth observed in db/db mice and humans with truncated LR (Bates et al., 2003; Clement et al., 1998), SOCS2 and CIS may also exert their influence on growth through regulation of LR signalling. Since LR Y1138S knock-in mice are not reduced in size (Bates et al., 2003) and since, in addition, no clear effect of CIS or SOCS2 was observed on leptin-dependent STAT3 signalling, this effect of SOCS2 and CIS probably occurs independently of STAT3. A good candidate is STAT5,
Materials and Methods

Construcsts

Generation of the mutant mouse LR(YF), LR(FYF), LR(FFY), LR(F3) in the pM377 expression vector was described elsewhere (Eyckerman et al., 1999). A pSEL-hEpoR-Y480 bait vector was derived from the described pSEL-hEpoR-Y402 LR, and identified a novel regulatory role for SOCS2. Full sensitive and flexible system for analysing interactions stimulation in vitro through the Y1077 and Y1138 positions since it is activated in different cell types upon leptin stimulation. Annealing of these oligonucleotide couples generated respectively an SacI and ligated in the pMET7 vector.

The pHEL-hEpoR-Y480 bait vector was derived from the described pHEL-hEpoR-Y402 bait construct most of the intracellular part of the LR was replaced by a flexible GSS linker. An unique EcoRI restriction site was introduced immediately following the JAK2 binding site through site-directed mutagenesis (Stratagene) using the primer pair 5’-GCTTGGTTGAGTGCTGGTGGT-3’ and 5’-GCTTGGTTGAGTGCTGGTGGT-3’. Phosphorylated oligonucleotides encoding two GSS repeats (5’-TCTTGTTGAGTGCTGGTGGT-3’ and 5’-AGACCCCATCCTGACC-3’) were annealed and head to tail ligated. Ligation was stopped by addition of two other annealed oligonucleotide couples: 5’-AACGTGGTTGAGTGCTGGTGGT-3’ and 5’-GGAGCCGAGCTGAGTGGGTGCTGATGTATAC-3’. This construct was then used to create the pMET7TAP2 construct using the primers 5’-GCGAGGAGCTCGAGCCAGCTCAGCAGCATTTCCAAGGGAAGGTTCGACGG-3’ and 5’-GCTTGGTTGAGTGCTGGTGGT-3’. Phosphorylated oligonucleotides encoding two GSS repeats (5’-GCTTGGTTGAGTGCTGGTGGT-3’ and 5’-AGACCCCATCCTGACC-3’) were annealed and head to tail ligated.

Expression of the gp130-fusion proteins, CIS and SOCS2, all flag-tagged, were verified in the TF-1 erythroleukaemia cell line grown in RPMI medium supplemented with 200 U/ml of recombinant human Epo. Western blot analysis was measured as described before.

Western blot analysis

Expression of the gp130-fusion proteins, CIS and SOCS2, all flag-tagged, were verified in the TF-1 erythroleukaemia cell line grown in RPMI medium supplemented with 200 U/ml of recombinant human Epo. Western blot analysis was measured as described before.
verified by western blot analysis. Transfected HEK293T or TF-1 cells were lysed in RIPA buffer: 200 mM NaCl, 50 mM Tris-HCl pH 8, 0.05% SDS, 2 mM EDTA, 1% NP40, Complete Protease Inhibitor Cocktail (Roche). 4X loading buffer (125 mM Tris-HCl pH 6.8, 6% SDS, 20% glycerol, 0.02% BFB, 10% β- mercaptoethanol) was added to the lysates which were then loaded on a 10% polyacrylamide gel and blotted overnight. Blotting efficiency was checked using PonceauS staining (Sigma). Flag-tagged proteins were revealed using monoclonal anti-Flag antibody M2 (Sigma) and anti-mouse-HRP (horseradish peroxidase) (Amerham Biosciences).

(Phospho)peptide affinity chromatography
Approximately 35X10^6 HEK293T cells were transfected with either pEF-FLAG-1mSOCS2 or pEF-FLAG-1mCIS and were lysed in lysis buffer (20 mM HEPES pH 7, 1 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 150 mM NaCl, 0.5% NP40, 20% glycerol, 1 mM NaVO4, Complete Protease Inhibitor Cocktail). The lysates were centrifuged for 5 minutes at 10,000 g and loaded on a pre-column with Sepharose 4B beads and streptavidin-agarose to prevent nonspecific interactions. Pre-cleared lysates were then incubated for 2 hours at 4°C with the (phospho)tyrosine peptides as indicated coupled to streptavidin-agarose beads through their biotin group. The beads were then washed twice with lysis buffer and resuspended in 2X loading buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.01% BFB, 5% β- mercaptoethanol). Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-flag antibody and anti-mouse-HRP. The sequences of the used peptides were biotin-QRQPSVK(P)Y985ATLVSNDK and biotin-HKPPQKTDV997FKSLK.

Co-immunoprecipitation
Approximately 2X10^6 HEK293T cells were transfected with pmet7-Flag-SOCS2 and pmet7-Etag-CIS. Cleared lysates (modified RIPA lysis buffer) were incubated with 4 μg/ml anti-FLAG mouse monoclonal antibody (Sigma) and protein-G-Sepharose (Amerham Biosciences). After immunoprecipitation, SDS-PAGE and western blotting, interactions were detected using anti-E-Tag antibody (Amerham Biosciences). We greatly acknowledge the technical support from Delphine Deveau for construction of the GGS baits, from Marc Goethals for peptide synthesis and from An Staes and Evy Timmerman for mass spectrometry analysis. This work was supported by grants from the Flanders Institute of Science and Technology (GOBU 010090 grant), from The Fund for Scientific Research – Flanders (FWO-V Grant number 1.5.446.98 to D.L., L.Z. and S.E.), from Ghent University (GOA 1201401) and from the Deutsche Forschungsgemeinschaft (SFB 542 to W.B.).

References


Cohen, B., Novick, D. and Rubinstein, M. (1996). Modulation of insulin activities by the NCBInr Database (taxonomy mammalia). Only spectra that exceeded Mascot’s threshold 100,000 were selected for fragmentation. For MS/MS analysis, an MS/MS gradient over 50 minutes of 0.1% formic acid in acetonitrile/water (7/3, v/v). Using a LC/Qq-TOF system (Agilent 6520) and the NCBInr Database (taxonomy mammalia). Only spectra that exceeded Mascot’s threshold score for identity (set at the 95% confidence level) were retained for further manual validation.

We greatly acknowledge the technical support from Delphine Deveau for construction of the GGS baits, from Marc Goethals for peptide synthesis and from An Staes and Evy Timmerman for mass spectrometry analysis. This work was supported by grants from the Flanders Institute of Science and Technology (GOBU 010090 grant), from The Fund for Scientific Research – Flanders (FWO-V Grant number 1.5.446.98 to D.L., L.Z. and S.E.), from Ghent University (GOA 1201401) and from the Deutsche Forschungsgemeinschaft (SFB 542 to W.B.).


CHAPTER 7: Leptin signalling in haematopoietic cells

I. Introduction

Leptin research was initially focused on its effects on hypothalamic weight regulation. During the last years however, leptin has been increasingly recognized as a cytokine-like hormone with pleiotropic actions on different physiological processes and in many peripheral tissues. Conceivably, leptin may function as a link between energy homeostasis and these physiological processes, since adequate energy stores are required to maintain certain energy-demanding processes. At present, the role of leptin in haematopoiesis is still a subject of much research and discussion. The functional long isoform of the LR is expressed in haematopoietic stem cells and in bone marrow stromal cells (Bennett et al., 1996; Cioffi et al., 1996; Konopleva et al., 1999). Involvement of leptin in modulation of haematopoiesis was demonstrated by colony formation studies where leptin stimulated the proliferation of stem cells and increased the numbers of lymphoid, erythroid and myeloid colonies (Bennett et al., 1996). Both ob/ob and db/db mice show a deficit in lymphopoietic progenitors and have defective erythrocyte production in the spleen, suggesting a role for leptin in the proliferation and expansion of haematopoietic stem cells and lymphoid progenitors (Bennett et al., 1996; Fantuzzi and Faggioni, 2000; Howard et al., 1999). Studies on signalling events induced by leptin in haematopoietic progenitor cells are very limited. Induction of the MAPK and PI3K pathways by leptin has been shown in peripheral blood mononuclear cells (Martin-Romero et al., 2000) and leptin was reported to prevent apoptosis of leukemia cells through STAT3 and MAPK activation (Tabe et al., 2004).

In order to gain more insight in the function of leptin as a modulator of haematopoiesis, we analysed leptin-mediated signalling events in haematopoietic cell lines using the MAPPIT technology. In preliminary experiments, no clear signals could be obtained with the classical STAT3-based MAPPIT set-up in several haematopoietic cell lines including erythroleukemic TF1, promyelocytic FDC-P1 and
prolymphocytic Ba/F3 cells. Since haematopoietic signalling depends predominantly on STAT5, we designed a variant of the MAPPIT method relying on functional complementation of STAT5 signalling. A novel prey construct was generated in which the gp130 domain is replaced by a STAT5 interaction sites-containing part of the βc-receptor. We used this novel βc-adaptation of the MAPPIT technology to examine leptin signalling in the TF1 and Ba/F3 haematopoietic cell lines. Several reported as well as novel interactions with the LR were identified. Our findings provide a basis for more detailed functional analysis of leptin signalling in haematopoiesis. Furthermore, MAPPIT proves to be a flexible method that can be adapted to other cell types like here for haematopoietic cell lines.

II. Article: Analysis of leptin signalling in haematopoietic cells using an adapted MAPPIT strategy.

Published in FEBS letters, 2006.

III. References

Analysis of leptin signalling in hematopoietic cells using an adapted MAPPIT strategy

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Abstract The adipocyte-secreted hormone leptin participates in the regulation of hematopoiesis and enhances proliferation of hematopoietic cells. We used an adaptation of the MAPPIT mammalian two-hybrid method to study leptin signalling in a hematopoietic setting. We confirmed the known interactions of suppressor of cytokine signalling 3 (SOCS3) and STAT5 with the Y985 and Y1077 motifs of the leptin receptor, respectively. We also provide evidence for novel interactions at the Y1077 motif, including phospholipase C gamma and several members of the SOCS protein family, further underscoring the important role of the Y1077 motif in leptin signalling.

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Keywords: MAPPIT; Leptin receptor; Signal transduction

1. Introduction

Leptins’ role has been studied most extensively in the central nervous system, where it regulates food intake and energy balance. Recent research revealed a wider spectrum of biological actions of this adipokine in other vital processes. Leptin-deficient (ob/ob) and leptin-receptor (LR)-deficient (db/db) mice develop a complex syndrome characterized not only by obesity and diabetic complications but also abnormal reproduction, hormonal imbalances and dysregulation of the hematopoietic and immune system [1]. Altered amounts of lymphoid progenitors, circulating lymphocytes, and leukocytes are observed in ob/ob and db/db mice, and correlation studies between plasma leptin concentration and leukocyte counts in humans led to the notion that leptin participates in the regulation of hematopoiesis [2–4]. In line with this, expression of the signalling-competent leptin receptor long isoform (LRlo) is observed in fetal liver, bone marrow, CD34+ progenitor cells as well as in several hematopoietic cell lines, such as TF1 and MO7E [5,6].

The LR, a member of the class I cytokine receptor family, is expressed as multiple alternatively spliced isoforms, of which only the LRlo can activate the JAK-STAT signalling pathway [7]. This LRlo form has three conserved tyrosine (Y) motifs in the intracellular domain, being Y985, Y1077 and Y1138 in the mouse LR. These sites become phosphorylated upon leptin treatment and are used as docking sites for several signalling or inhibitory molecules. Examples include recruitment of STAT3 (signalling transducer and activator of transcription) via the Y1138 motif of the LR, STAT5 via the Y1077 and Y1138 motif of the LR [8] and binding of the SH2-containing phosphatase SHP-2 on the Y985 motif leading to activation of the Ras-MAPK pathway [9]. In addition, several members of the SOCS (suppressor of cytokine signalling) protein family can interact with the LR. SOCS3 associates predominantly with the pY985 motif [10,11] and was identified as a potent inhibitor of LR signalling. SOCS2 interacts with Y1077 and cytokine inducible SH2 containing protein (CIS) with both Y985 and Y1077 motifs (Lavens et al., in press).

In this report, we used the human premyeloid TF1 and the murine Ba/F3 pro-B cell lines as models to study protein interactions with the LR in a hematopoietic environment. To this end, we adapted the MAPPIT (mammalian protein–protein interaction trap) method [12] to operate in hematopoietic cell types.

2. Materials and methods

2.1. Constructs

Generation of the mutant mouse LR(YYF) in the pMET7 expression vector was described elsewhere [13]. Construction of the pSEL-60GGS-mLR-Y985 and pSEL-60GGS-mLR-Y1077 baits was described in Lavens et al. (in press). A fragment of the intracellular part of the hJc receptor, containing 4 tyrosine motifs, was amplified by PCR on the pSV532-hJc plasmid using primer 1 and primer 2 (see Table 1). The PCR fragment was digested with Apol and was ligated into the pMG2-SVT plasmid [12] which was digested with EcoRI leading to replacement of the gpl30 part by the pMG2-construct. This prey-construct was called pMjc-SVT. This construct was further digested with EcoRI and NotI, allowing exchange of the SVT part by a full-length rat SOCS3 F25A PCR fragment (primers 3 and 4). The KIR domain of SOCS3 was rendered inactive by a F25A mutation that was introduced using primers 5 and 6. All other members of the SOCS family were cloned as pMjc-precy fusion proteins. Murine CIS and SOCS2 were already cloned as pMG2-preys [14], and were transferred as EcoRI–XhoI fragments. Murine SOCS1 was amplified by PCR using primers 7 and 8 and was ligated into EcoRI–NotI opened pMjc vector. Inactivation of the KIR domain of SOCS1 was done with primers 9 and 10 leading to a F99A mutation. The pMjc–mSOCS4 construct was obtained by PCR on cDNA prepared from mouse thymus (gift from Dr. Peter Brouckaert) with primers 11 and 12 and insertion in the EcoRI–NotI opened pMjc vector. The pMjc–mSOCS5 and -mSOCS6 prey constructs were generated by amplification from the

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2.2. Cell lines, electroporation conditions, reporter assays, expression in independent experiments.

ulated/non-stimulated or NS) and are representative of at least three

2.3. Interaction of signalling molecules with the LR

3. Results

3.1. βc-MAPPIT: design and proof of principle

The MAPPIT concept is based on JAK-STAT signalling and is shown in Fig. 1. We here designed a variant of the MAPPIT method that enables us to detect protein interactions in hematopoietic cells. Since cytokines predominantly activate STAT5 in hematopoietic cells, we designed novel prey constructs, wherein the gp130 moiety is swapped for a part of the βc-receptor, containing several STAT5 interaction sites. In the MAPPIT bait constructs, we replaced the cytosolic domain of the LR following the JAK2 interaction site, by a large array of Gly-Gly-Ser (GGS) repeats (Fig. 1B) in order to prevent any background prey association with the LR-F3 (Lavens et al., in press). Interaction of co-transfected GGS-bait and βc-prey will lead in this setting to activation of the STAT5-dependent SPI2.1-luciferase reporter. The MAPPIT technique also allows the analysis of interactions with the LR itself. A mutant LR(YYF) [13] was used to avoid possible background by activation of STAT3 and 5 via Y1077 (Fig. 1C). Binding of STAT5 to the YYF motif [18], did not interfere with the read-out in the hematopoietic cell systems used.

We initially tested the concept using a SOCS3(F25A)-prey. SOCS3 strongly binds to the LR pY985-motif and negatively regulates LR signalling [18]. To suppress its inhibitory effect on JAK2 activation, we inactivated the Kinase Inhibitory Region (KIR domain) in the SOCS3 prey by introducing a F25A mutation. SV40 large T protein (SVT) was used as negative prey control. As shown in Fig. 2, clear, specific signals were obtained in both Ba/F3 and TF1-M1-16 cells with either the LR(FYF) lacking the Y1138 STAT recruitment motif or with the F3-prey association with the LR-F3 (Lavens et al., in press). Activation of the pGL2-SPI2.1-luciferase reporter. The MAPPIT technique also allows the analysis of interactions with the LR itself. A mutant LR(YYF) [13] was used to avoid possible background by activation of STAT3 and 5 via Y1077 (Fig. 1C). Binding of STAT5 to the YYF motif [18], did not interfere with the read-out in the hematopoietic cell systems used.

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<table>
<thead>
<tr>
<th>Primer</th>
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<th>Reverse</th>
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Table 1
Overview of primers used in this study
used in this study were full-length STAT5a and part of PLCγ encompassing its two SH2-domains. The STAT5a prey was made signalling-deficient by introducing a Y694F mutation. Strong luciferase signals were detected with the PLCγ-prey, and a weaker interaction was seen with the STAT5a-prey (Fig. 3A). Analysis using the GGS-LR Y985 and GGS-LR...
Y1077 baits revealed that both preys only interact with the Y1077 motif (Fig. 3B). We confirmed the PLCγ dataset with (phospho)peptide affinity chromatography using lysates from Hek293T cells containing a FLAG-tagged PLCγ-derived poly-peptide encompassing both SH2 domains. Clear binding was observed with the pY1077 phospho-peptide, with only minor binding to the pY985 motif. No detectable binding was observed with the non-phosphorylated peptides (Fig. 4B).

3.3. Interaction map of SOCS proteins with the LR

To determine their interaction with the LR, all members of the SOCS family were cloned as βc-preys and were tested for interaction with LR(YYF) in Ba/F3 or TF1-M1-16 cells (Fig. 4A and B). The inhibitory effect by SOCS1 was suppressed by a F59A mutation in its KIR domain, similar to SOCS3 (see above). In Ba/F3 cells, clear induction of luciferase activity indicated that CIS, SOCS2 and SOCS3 interact with the LR(YYF), consistent previous reports [11] (Lakens et al., in press). In addition, reproducible induction of luciferase activity was also seen with SOCS6 and SOCS7. Similar results were obtained in TF1-M1-16 cells. No signal was obtained in the case of the βc-SOCS1 F59A, SOCS4 and SOCS5 preys.

A detailed binding analysis of SOCS preys showed that SOCS3 interacted with the Y985 motif, while SOCS2 and -6 bind to the Y1077 motif. CIS and SOCS7 can interact with both motifs (Fig. 4C). These βc-preys bind specifically with the tyrosine motifs of the LR, since no interaction could be found with an irrelevant GGS-FKBP12 bait and replacement of preys by a wild-type SOCS protein (SOCS7) did not lead to luciferase activity (data not shown).

4. Discussion

Although a role of leptin in hematopoiesis is now well documented, studies on the intracellular pathways used by the LR in hematopoietic cells are limited. Leptin can prevent apoptosis of leukaemia cells through STAT3 and mitogen-activated protein kinase (MAPK) phosphorylation [19] and activation of the MAPK and PI3-K pathways by leptin has been shown in peripheral blood mononuclear cells [20]. Here, we adapted the MAPPIIT mammalian two-hybrid method to a hematopoietic setting and studied protein interactions with the LR in TF1-M1-16 and Ba/F3 cells. The basic MAPPIIT set-up using STAT3 did not function in these cell lines, presumably due to low amounts of endogenous STAT3. We therefore swapped the gp130 moiety in the prey constructs for part of the cytosolic domain of βc capable of recruiting and activating STAT5, which is highly expressed in hematopoietic cell types. Our experiments illustrate the flexibility of the MAPPIIT strategy, which can be adapted to operate in different types of human cells by simple adjustment to different reporter systems.

The murine LR cytoplasmic tail contains three conserved tyrosine motifs at positions Y985, Y1077 and Y1138. With the βc-MAPPIIT approach we confirmed the interaction of SOCS3 with the LR Y985 motif in hematopoietic cells, whilst only a very weak effect was seen with Y1077, consistent with the weak interaction with this motif (Figs. 2 and 4C) (10). The role of the Y1077 motif in leptin signalling is still somewhat controversial since its tyrosine phosphorylation is difficult to demonstrate. However, the Y1077 motif was shown to interact with SOCS3 and STAT5 in a phosphorylation-dependent modus [8,11]. Here, we provide further evidence for an important role of the Y1077 motif in leptin signalling with several additional signalling proteins interacting at this site. Although there is evidence suggesting involvement of the phospholipase C–phosphokinase C (PLC–PKC) pathway in leptin signalling [21–23], no proof for interaction of PLC proteins with the LR was obtained so far. We here show that PLCγ can interact via its SH2 domains with the LR(YYF) and more detailed analysis using both βc-MAPPIIT and peptide affinity chromatography revealed that PLCγ interacts with the phosphorylated Y1077 motif.
Since SOCS3 and SOCS7 were reported to inhibit leptin signalling in different ways [24], we performed a MAPPIT scan to monitor the binding capacity of all SOCS proteins with the LR. We were able to detect binding of SOCS3 as expected, but also of CIS, SOCS2, SOCS6 and SOCS7. SOCS4- and SOCS5-preys do not induce reporter activity, indicating that no interaction occurs, although direct inhibition of STAT5 activation cannot be completely ruled out. Also, no interaction of SOCS1 is detected, which is somewhat surprising since SOCS1 is known to bind JAK2 [25] and can inhibit leptin signalling in cell-based assays (Piessevaux et al., submitted for publication). Possibly, certain preys that directly interact with JAK2 may be subject to a topological restraint whereby phosphorylation of the prey tyrosines by the JAK is sterically impossible. Interestingly, the closely related SOCS proteins CIS and SOCS2 both bind to the Y1077 motif, but only CIS appears to interact with the Y985

Fig. 3. Interaction of STAT5 and PLCγ with the LR. (A) Ba/F3 (Bars 1–3) or TF1-M1-16 cells (Bars 4–6) were transiently electroporated with plasmids encoding pMet7-LR(YYF) and a βc-prey as indicated or an empty vector, combined with the pGL2-SPI2.1-luciferase reporter. Experimental set-up was as in Fig. 2. The values were normalized for background as mentioned in Fig. 2. (B) Ba/F3 were transiently electroporated with plasmids encoding the pSEL60GGS-LR Y985 or pSEL-60GGS-LR Y1077 bait and a βc-prey as indicated or an empty vector, combined with the pGL2-SPI2.1-luciferase reporter. Experimental set-up was as in Fig. 2. (C) The FLAG-tagged PLCγ(2x SH2) protein was expressed in Hek293T cells and total cell lysates were incubated with biotinylated peptides encompassing the LR (phosphorylated) Y1077 or Y985 motifs. Immunoblotting with anti-FLAG antibody revealed specific interaction of PLCγ with the phosphorylated Y1077 motif.
motif. Similar data were obtained in non-haematopoietic cell types (Lavens et al., in press). Similarly, SOCS7 can interact with both the Y985 and the Y1077 motifs, whilst SOCS6 only binds Y1077. Inhibition or binding of SOCS6 on the LR has not been reported so far, although both can be expressed in hematopoietic cells [26]. Inhibition of leptin signalling by SOCS7 has been shown through binding and inhibition of STAT3 by SOCS7 [24]. However, binding of SOCS7 on the LR itself was not demonstrated before. Our findings suggest that besides the well-established inhibitory effect of SOCS3 on hypothalamic leptin signalling, other SOCS proteins can bind to the LR and possibly may modulate leptin signalling in other, e.g., peripheral cell types.

Together, our findings illustrate that the MAPPIT strategy can easily be adapted to hematopoietic cell types. Our findings identify several novel interactions with the LR and provide a rationale for more detailed functional analysis of leptin signalling in hematopoiesis.
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References

CHAPTER 8: Modulation of leptin receptor signalling.

I. Review: Negative regulation of leptin receptor signalling

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Review: negative regulation of leptin receptor signalling

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ABSTRACT. Leptin was discovered as an adipostat, regulating body weight by balancing food intake and energy expenditure. Recently, leptin emerged as a pleiotropic cytokine. It plays a substantial role in a wide spectrum of other functions including immune regulation, bone formation and fertility. Leptin signalling is under tight control. Aberrations of this stringent control system may be implicated in a variety of pathologies. Here, we review the various mechanisms that control cellular leptin receptor signalling.

Keywords: leptin receptor signalling, negative regulation, SHP-2, PTP1B, SOCS proteins

Leptin plays a major role in the regulation of energy homeostasis and food intake. It is mainly produced in white adipose tissue and, to a lesser degree, in the stomach and in some other tissues [1, 2]. Leptin is released into the circulation and is translocated through the blood-brain-barrier (BBB) to target the leptin receptor (LR) in the hypothalamus. Functioning as an adipostat, it signals the state of body fat reserves to the brain. Aberrations in leptin signalling are often associated with obesity, but only a minority of obese individuals show a deficiency in leptin or its receptor. Instead, most cases of human obesity show a state of relative leptin resistance, as reflected in high serum leptin levels [3, 4]. This resistance may be situated at different levels in the leptin pathway, including saturation of transport through the blood-brain barrier, aberrations in LR signal transduction or downstream effects on neural networks in the hypothalamus [5, 6].

Leptin is a pleiotropic cytokine. Apart from its role in energy homeostasis, it is also implicated in a range of other, often peripheral processes, including immune response, bone formation, angiogenesis and reproduction. Recent findings suggest that leptin is involved in a variety of pathological processes, including cardiovascular and autoimmune diseases [7, 8].

Given leptin’s wide range of important functions, its activities must be under stringent control. In this review we discuss the molecular mechanisms that are responsible for the modulation of signal transduction via the LR. A schematic representation of LR signalling and modulation is shown in figure 1.

JAK-STAT SIGNALLING

At least 5 different LR isoforms exist, but the main player responsible for signal transduction is the long isoform of the LR [9]. Canonical leptin signalling occurs through the JAK-STAT pathway. Ligand binding results in LR clustering, bringing the associated JAKs (janus kinase) into close proximity. This allows them to activate each other by cross-phosphorylating tyrosines in their activation loop. These activated JAK kinases then phosphorylate tyrosines in the cytoplasmic tail of the receptor and on the JAKs, forming docking sites for signalling proteins. Amongst these, the STATs (signal transducers and activators of transcription) associate with the phosphotyrosines in the receptor via their SH2 domain and become activated by JAK2 mediated tyrosine phosphorylation. The activated STATs then dissociate from the receptor and translocate to the nucleus as dimers to induce specific target genes.

JAK2 is constitutively associated with the membrane proximal box1 in the cytoplasmatic tail of the LR [10, 11]. The intracellular part of the receptor also carries three conserved tyrosines at positions Y985, Y1077 and Y1138 (murine numbering). The membrane distal tyrosine is embedded in aYXXQ motif and is responsible for the recruitment of STAT3 [12, 13]. STAT3 activation was demonstrated after leptin stimulation in the hypothalamus of mice [14]. Knock-in mice containing an Y1138S mutation are incapable of STAT3 activation and reveal a severely obese phenotype. They do not show the infertility and reduced size that is seen in db/db mice that are truncated in the long LR, indicative of the involvement of other signal transducers [15]. Leptin-induced activation of STAT1 and STAT5B, in addition to STAT3, was shown in COS cells and in HIT-T15 cells [16, 17]. In the latter cell line, STAT1 was activated via Y1138 while STAT5B activation occurred via both Y1138 and Y1077 [17].

Next to JAK-STAT signalling, leptin also activates other pathways. A number of adaptor molecules can associate with the receptor and link to several signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway (see below) and the phosphoinositol 3-kinase
(PI3K) pathway. In the latter, the JAK2-interacting protein, SH2-B, mediates binding of IRS (insulin receptor substrate) proteins that function as adaptors for PI3K [18, 19]. PI3K transforms phosphatidylinositol4,5-biphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3) eventually resulting in reduced levels of cAMP. It was also demonstrated that leptin has an inhibitory role on hypothalamic AMPK (AMP-activated protein kinase) activity which contributes to body weight regulation [20].

MODULATION OF FUNCTIONAL RECEPTOR EXPRESSION

Obviously, receptor internalisation is an effective mechanism for rapidly turning off cytokine signalling. Upon ligand binding, cytokine receptors can be internalised via the clathrin-coated pit pathway into early endosomes. Trafficking dynamics of the LR with receptor internalisation and subsequent degradation or recycling back to the cell surface clearly are involved in the regulation of leptin signalling. In steady-state conditions, no more than 25% of the LR is located at the cell surface, whilst the majority of the LR are found in intracellular pools [21]. This distribution of the LR may be explained by its tendency to constitutive endocytosis resulting in short-lived membrane expression. In addition, some of the newly-synthesized LRs are retained intracellularly based on a retention signal in the transmembrane domain [22]. Whether external stimuli modulate this LR localisation throughout the cell and in this way regulate leptin sensitivity remains to be determined.

\[^{125}\text{I}\]-labeled leptin uptake experiments demonstrated that LRs are also internalized upon ligand binding via clathrin-mediated endocytosis leading to leptin degradation in the lysosomes [21, 23]. An internalisation signal was identified in the intracellular part of the receptor in immediate proximity to the membrane [23]. Compared with other LR splice variants, the long LR isoform seemed to be depleted relatively quickly from the cell surface upon leptin exposure, suggesting it is most sensitive to leptin-induced down-regulation while its limited recycling to the cell membrane was slow [21-24]. This favoured down-modulation of LR signalling may be implicated in leptin resistance [25, 26].

Recently, it was demonstrated that both the long LR and the short LR, a membrane-anchored isoform with a short cytoplasmic tail, become ubiquitinated. Unlike for the long LR, this ubiquitination is essential for clathrin-mediated endocytosis of the short LR [27]. Many aspects of the mechanisms underlying LR cell surface expression and internalisation remain to be elucidated. It is likely that additional proteins involved in ubiquitination of the (activated) LR complex remain to be identified.

A soluble form of the LR associates with circulating leptin [28]. Secreted cytokine receptors can protect their ligands...
from either degradation or clearance and thus significantly extend their half-life or they can act as antagonists, capturing their ligand and thus preventing signalling by their membrane-spanning counterparts. In mice, the soluble LR is generated by alternative mRNA splicing. In contrast, no such mRNA splice variant has been discovered in humans; a secreted human LR is generated by ectodomain shedding of membrane-anchored LRs including the signalling long form, by a hitherto unknown protease [29-31]. Although the soluble LR appears important for keeping leptin available in circulation, it is at the same time, capable of competing with the long LR isoform for leptin binding and may suppress leptin action in that way [32-35]. This could indicate that the secreted LR plays an important role in determining leptin levels available for signal transduction. It is of note that the relative concentrations of the soluble LR and free leptin are similar, while in obese individuals concentrations of free leptin exceed by far the concentrations of secreted LR [36].

PHOSPHATASES

SH2 domain-containing phosphatase-2 (SHP-2) is a constitutively expressed protein tyrosine phosphatase known to be involved in the dephosphorylation of the JAKs. It carries two tandem SH2 domains followed by a tyrosine phosphatase catalytic domain and associates directly with the LR at position Y985 [37]. The exact role of SHP-2 in LR signalling has been a long standing matter of debate. Despite its initial identification as an inhibitor of LR signalling (see below), it also appeared as a strong activator of the MAPK pathway. ERK activation occurs predominantly via SHP-2 recruitment to tyrosine Y985 via its C-terminal SH2 domain. SHP-2 is phosphorylated by JAK2 and forms a docking site for the adaptor protein growth factor receptor binding 2 (Grb2) leading to the activation of the ERK signalling cascade [12]. Alternatively, ERK is also directly activated by JAK2, but still requires the intervention of SHP-2 [38]. Leptin-triggered activation of MAPK was observed both peripherally and centrally. Recently, regulation of calcium fluxes involving MAPK activity was shown in lateral hypothalamic neurons upon leptin stimulation [39]. Also, NO (nitric oxide) production induced by leptin via MAPK activation was observed in white adipocytes [40]. Moreover, leptin induced MAPK is involved in full activation of the DNA binding of STAT3 by mediating serine phosphorylation at position S727 of STAT3 [41].

On the other hand, many reports have also attributed an inhibitory role to the SHP-2 phosphatase in LR signalling. Mutation of the Y986 position in the human LR led to augmented STAT3 signalling, and inhibitory properties associated with this position were ascribed to the negative regulatory function of SHP-2 [42]. However, suppressor of cytokine signalling 3 (SOCS3), identified as a strong inhibitor of LR signalling (see below), was found to interact with the corresponding Y985 position in the murine LR [43-45]. SOCS3 is part of the SOCS family and its inhibitory mechanism is discussed below. SHP-2 and SOCS3 have very similar binding specificities, and overlapping binding sites were also observed for the gp130 chain [46-49]. Thus, the negative regulation associated with the membrane proximal tyrosine position is partly attributed to SOCS3. However, SHP-2-mediated dephosphorylation of JAK2 was demonstrated in vitro [37]. Recently, forebrain-specific SHP-2-deficient mice revealed that SHP-2 moderately down-modulates JAK2 and STAT3 activation in vivo [50]. Although SHP-2 has a modest role in terminating leptin signal transduction, its dominant induction of the ERK pathway makes it overall an enhancer of leptin signalling, whereby it may function as a switch towards MAPK signalling.

Protein tyrosine phosphatase 1B (PTP1B) is a crucial protein tyrosine phosphatase implicated in the negative regulation of leptin receptor signalling. PTP1B deficiency results in hypersensitivity to insulin and leptin in mice, and leads to protection from high fat diet obesity [51]. PTP1B harbours two phosphotyrosine binding pockets in its catalytic domain that determine its intrinsic specificity. A consensus substrate recognition motif was found in the kinase activation loop of the insulin receptor and in JAK2 [52-54]. Both in vivo and in vitro data demonstrate that PTP1B targets LR signalling predominantly by dephosphorylating JAK2 [55-58]. PTP1B is a negative mediator of both the JAK-STAT and MAPK pathway in leptin receptor signalling. PTP1B-mediated hypophosphorylation of JAK2 in a mouse hypothalamic neuronal cell line abrogated the leptin-dependent induction of the STAT3 and MAPK inducible SOCS3 and c-fos genes, respectively [56]. Recently, leptin induced PTP1B was observed in liver, raising the possibility that PTP1B may also function in a negative feedback loop [59]. Diet-induced obesity is associated with increased hepatic PTP1B levels. Aberrant PTP1B activity is implicated in leptin resistance and PTP1B is currently being investigated as a drug target in obesity [60-63]. PTP1B is localized predominantly on the ER (endoplasmic reticulum) via its C-terminal hydrophobic targeting sequence [64]. How PTP1B acts on its substrates remains unclear. It was demonstrated that the platelet-derived growth factor (PDGF) receptor becomes dephosphorylated by PTP1B at the ER after internalization [65]. Recently, direct interaction of PTP1B with the insulin receptor was observed in a perinuclear endosome compartment [66]. On the other hand, it has been demonstrated that internalisation of the insulin receptor is not essential for interaction with PTP1B and subsequent dephosphorylation [67]. In line with this, proteolytic cleavage of PTP1B can lead to the relocalization of the catalytic domain of PTP1B to the cytosol [68].

The ubiquitously expressed phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumour suppressor protein and its mutation is linked with several human cancer types [69]. It belongs to the family of protein tyrosine phosphatases but also possesses lipid phosphatase activity. PTEN suppresses the PI3K pathway by hydrolyzing the secondary messenger PIP3 back to PIP2 [70]. It was demonstrated that hypothalamic PI3K is involved in leptin-induced reduction in food intake [19]. Surprisingly, specific disruption of PTEN restricted to the hypothalamic neurons expressing the anorexigenic proopiomelanocortin (POMC) neuropeptide results in an obese phenotype associated with leptin resistance [71].

SUPPRESSORS OF CYTOKINE SIGNALLING

The family of SOCS proteins consists of 8 members: cytokine inducible SH2 protein (CIS) and SOCS1 through SOCS7. SOCS proteins have a characteristic domain
SOCS3-deficient mice that die model, elevated levels of SOCS3 were found [43]. Unlike rons in the hypothalamus [77]. In these hypothalamic functional marker for identification of leptin-sensitive neuro-
tion in leptin-deficient ob/ob mice [12, 43, 76]. SOCS3 is a
SOCS3 was identified as a potent inhibitor of LR signal-
ing possible cross-regulation among cytokine systems.
Leptin induces SOCS3 expression in a rapid and transient manner while CIS expression accumulates over a longer period of time [43, 73, 74]. A role for leptin has also been implicated in the expression of SOCS1 and, to a lesser extent of SOCS2 [74, 75].
SOCS3 was identified as a potent inhibitor of LR signalling [43]. Its STAT3-mediated expression is induced in the hypothalamus and liver after peripheral leptin administra-
tion in leptin-deficient ob/ob mice [12, 43, 76]. SOCS3 is a functional marker for identification of leptin-sensitive neu-
rons in the hypothalamus [77]. In these hypothalamic neurons of the leptin-resistant lethal yellow (Ay/a) mouse model, elevated levels of SOCS3 were found [43]. Unlike SOCS3-deficient mice that die in utero, SOCS3 haploin-
sufficient or neural-cell specific-deficient mice are viable and show augmented leptin sensitivity in the hypothala-
mus and a remarkable attenuation of diet-induced obesity [78, 79]. It was demonstrated that SOCS3 action is in-
volved in rendering the LR refractory to reactivation after chronic leptin stimulation [80]. These observations show SOCS3 up as a key mediator of negative regulation of leptin signalling and suggest a prominent role in leptin resistance.

Only SOCS1 and 3 carry a KIR domain in their N-terminal region involved in direct inhibition of the JAK kinase activity. They both inhibit leptin receptor signalling, using a slightly different mechanism. SOCS1 directly interacts with the kinase domain of JAK2 by targeting the phospho-
tyrosine at position Y1007 in the activation loop of JAK2 [81, 82]. The KIR domain is essential for the inhibitory function of the SOCS protein [82]. It associates with the catalytic groove of JAK2 and is suggested to act as a pseudosubstrate which mimics the activation loop that regulates access to the catalytic groove [81, 82]. It may obstruct the ATP binding pocket and hinder accessibility for substrates [81, 82]. Unlike SOCS1, SOCS3 has only weak affinity for JAK2. It is thought to inhibit the kinase activity through its KIR domain after binding via its SH2 domain with phosphotyrosine motifs in the receptor in close proximity to the JAKs [83]. Indeed, SOCS3 associates with the LR at the membrane proximal tyrosine Y985 domain [44, 84]. It also weakly binds the highly similar Y1077 interaction site, with an accessory effect on LR signalling inhibition [84].

Using the MAPPIT technique, a two-hybrid method based on cytokine signalling, we recently demonstrated the inter-
action of CIS and SOCS2, two other members of the SOCS protein family, with the LR [45, 74]. We showed that CIS interacts with the two membrane proximal tyrosine motifs at positions Y985 and Y1077, while SOCS2 only associ-
ated with the latter of the two. Phosphotyrosine specific interaction of SOCS2 with the LR Y1077 motif was con-
firmed by peptide affinity chromatography (PAC). Using this method, we also demonstrated that SOCS2 binds specifically to the phosphotyrosine Y1138 peptide. An overview of LR/SOCS interactions is given in table 1. Interactions with the LR Y1138 motif and those involving SOCS1 were only analysed using PAC since in these cases interference occurs with the MAPPIT read-out. Of note, MAPPIT proved to be a highly sensitive technique that can detect weak or transient (but functionally relevant) interac-
tions that could not be detected by PAC. CIS and SOCS2 are known inhibitors of STAT5 activation. Although negative regulation of a leptin-induced STAT3 binding reporter gene by CIS was suggested, we did not

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**Figure 2**

Schematic overview of SOCS protein structure. The KIR domain is indicated with a black box, the C-terminal, conserved tyrosines are represented by a black line.
observe any inhibitory effect on STAT3-mediated LR signalling by either CIS or SOCS2 [73, 74]. Instead, we suggest an inhibitory role in leptin-induced STAT5 signalling through interference with STAT5a recruitment to the Y1077 tyrosine motif in a MAPPIT based experiment [74]. Supporting this notion, SOCS2 binding completely overlaps with STAT5 association at the LR. CIS and SOCS2 may be implicated in preventing recruitment of downstream signalling moieties to the LR. Both SOCS2 knockouts and CIS transgenics show growth abnormalities, the former being larger and the latter smaller than normal [85, 86]. Although both SOCS proteins are negative regulators of GH signalling, growth retardation in people with a truncated LR as well as in LR null db/db mice suggests these SOCS proteins may additionally influence growth via the LR [15, 87]. Leptin has been identified as a proinflammatory cytokine [88]. It is implicated in the pathogenesis of several autoimmune diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease [7, 8]. A role for leptin was described in T-cell proliferation and switching towards a Th1 response [89]. CIS transgenic mice exhibit a shift to activation of Th2 cells [85], an effect that may, in part, be explained by its effect on leptin signalling in T-cells. More detailed analysis in cell-type specific expression and function will be needed to elucidate the specific roles of SOCS proteins in leptin signalling. Possibly, different physiological functions of leptin may be under the control of different SOCS proteins.

More detailed examination of the binding modalities of SOCS proteins with the LR reveals that the SOCS-box of CIS is implicated in the association with the LR (Lavens et al., in press). The conserved C-terminal tyrosine at position Y253 is essential for binding to both membrane proximal tyrosines. The same phenomenon is also observed for interaction with other cytokine receptors such as the EpoR but not for association with the unrelated MyD88 protein, an adaptor protein involved in toll-like receptor (TLR) signalling [74, 90]. In contrast, the corresponding C-terminal tyrosine or even the entire SOCS-box of the highly related SOCS2 protein are not essential for interaction with the LR, and deletion of the SOCS-box also, hardly influenced the inhibitory capacity of SOCS1 or SOCS3 on LR signalling [74]. This indispensable role of the SOCS-box for binding with the LR (and likely other cytokine receptors as well), is probably an exclusive characteristic of CIS. The exact functional role of the C-terminus of CIS is still unclear. This observation is very reminiscent of the Von Hippel-Lindau protein whereby the C-terminus of its SOCS-box is also involved in substrate recognition [91, 92].

Recently, it has become clear that regulation by certain SOCS proteins can be more complex than a mere negative feedback loop. It has been demonstrated that, apart from its negative regulatory effects, SOCS2 can also have positive effects on cytokine signalling, as was clearly observed in vivo and in vitro for GHR signalling [93, 94]. SOCS2 interference with other SOCS proteins has been observed in several cytokine receptor systems including LR signalling [74, 93, 95, 96, 97]. We recently demonstrated that SOCS2 interferes with the association of CIS to the membrane proximal tyrosine of the LR, although no direct binding of SOCS2 with this tyrosine position was demonstrated [74]. In addition, SOCS2 can impair the inhibitory effect of SOCS1 or SOCS3 on leptin-induced signalling. This effect strictly relied on the presence of the SOCS-box of both SOCS-proteins, since deletion of the SOCS-box of either SOCS2 or SOCS1 and SOCS3 abolished complete SOCS2 interference [97]. SOCS2 is demonstrated to associate with all members of the SOCS protein family [74, 96, 97]. Abolishing the elonginB/C recruitment potential of SOCS2 has no effect on its SOCS interaction capacity but leads to complete loss of its functional interfering characteristics [74, 97]. SOCS2 influences the stability of target SOCS proteins and this effect is sensitive to proteasome inhibitors and clearly relies on the presence of its BC-box [96, 97]. Together, these data strongly suggest that SOCS2 can target SOCS proteins for degradation and regulate SOCS protein turnover. In addition, we demonstrated that SOCS6 and SOCS7 are also capable of interacting with the SOCS protein family members. Similar potentiating effects as with SOCS2 are observed for SOCS6 in LR signalling as well as other cytokine receptor systems [97]. This cross-regulatory effect of SOCS proteins may be of great importance in restoring cellular sensitivity after cytokine stimulation. Indeed, it has been reported that the expression of SOCS2 is in many cases more prolonged than that seen for other SOCS proteins [96-100].

Using the MAPPIT methodology, we recently demonstrated that SOCS6 and SOCS7 also interact with the LR. Both associate with the Y1077 motif whilst only SOCS7 interacts with the more membrane proximal tyrosine [101]. It was reported that SOCS7 is implicated in LR signalling termination. It can inhibit STAT3 activation which we speculate may involve LR association, but it can also interact with activated STAT3 molecules to prevent them from translocating to the nucleus [102].

### Table 1

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Binding of the SOCS proteins, CIS and SOCS1 through SOCS3, with the tyrosines of the LR based on peptide affinity chromatography (PAC) with corresponding phosphorylated and non-phosphorylated tyrosine motifs and based on mammalian protein-protein interaction trap (MAPPIT) [74, 84, 100].
CONCLUSION

Leptin is involved in a variety of crucial processes including adipocyte metabolism and immune responses, and aberrant leptin signalling has been implicated in several pathophysiological processes. Tight control mechanisms exist that regulate leptin receptor signal transduction. Today, SOCS3 and PTP1B are the two molecules that are most associated with modulation of LR signalling. However, the involvement of other mechanisms and molecules, especially other SOCS proteins is emerging. It is likely that the different inhibitory molecules may be implicated in the regulation of leptin functions in different cell types. Further investigation will be needed to clarify the complex regulatory mechanisms that control leptin receptor signaling in many vital processes.

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REFERENCES


Review: negative regulation of leptin receptor signalling


43. Salmeen A, Andersen JN, Myers MP, Tonks NK, Barford D. Molecular basis for the dephosphorylation of the activation segment of the insulin receptor by protein tyrosine phosphatase 1B. Mol Cell 2000; 6: 1401.


63. Tonks NK. PTP1B. from the sidelines to the front lines! FERS Lett 2003; 546: 140.

64. Frangioni JV, Braham PH, Shifrin V, Jost CA, Neel BG. The non-transmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. Cell 1992; 68: 545.


Part III : The many faces of the SOCS box
Chapter 9: Enhanced cytokine signalling by SOCS box-dependent cross-regulation between SOCS proteins.

I. Introduction

Increasing evidence indicates that SOCS molecules not only act as inhibitors of cytokine responses but also play an essential role in determining cell fate by exerting broader regulatory mechanisms. SOCS proteins were for example shown to exert a stimulating effect on signal transduction of the RAS/MAPK pathway. In this respect, CIS increases ERK- and JNK-mediated MAPK signalling in activated T cells (Li et al., 2000). Tyrosine phosphorylation of the SOCS box of SOCS3 allows interaction with the Ras inhibitor p120RasGAP, resulting in sustained ERK activation (Cacalano et al., 2001). SOCS2 expression enhances ERK (and STAT) phosphorylation following cytokine treatment (Johnston, 2004). Also the Drosophila SOCS44A protein can have a stimulatory action on the EGFR/MAPK pathway (Rawlings et al., 2004). Additionally, positive effects were reported for SOCS2 and SOCS6 in other signalling cascades. SOCS6 increases AKT activation upon insulin stimulation possibly by interacting with the p85 monomer which is an attenuator of PI-3K dependent pathways. Accordingly, an improvement in glucose metabolism was observed in SOCS6 transgenic mice (Li et al., 2004). In vitro data demonstrated a dual effect of SOCS2 on GH responses with low concentrations having a suppressive effect while signalling is restored and even enhanced at higher concentrations (Favre et al., 1999). This paradoxal effect of SOCS2 is supported by in vivo data since deficiency and overexpression of SOCS2 in mice causes a similar phenotype characterized by enhanced growth (Greenhalgh et al., 2002; Metcalf et al., 2000).

In this paper we examined the molecular mechanisms underlying the positive effect of SOCS2 on cytokine signalling and show that SOCS2 can interfere with the inhibitory functions of SOCS1 and SOCS3 in GH, type I IFN and leptin signalling.
The modalities of this interfering effect were studied in greater detail. Using MAPPIT we found that SOCS2 (and SOCS6 and -7) can bind with all members of the SOCS protein family and the SOCS box of the targeted SOCS appears to be implicated. In analogy to the SOCS2 interference with CIS interaction at the LR described in chapter 6, this regulatory capacity of SOCS2 depends on Elongin B/C recruitment to its SOCS box. We observed SOCS2 mediated degradation of SOCS1 but not by its Elongin B/C recruitment-deficient mutant, suggesting that SOCS2 targets other SOCS proteins for proteasomal degradation. The cross-regulatory mechanism between SOCS molecules may be important for the restoration of cellular responsiveness for subsequent stimulation by eliminating excess SOCS levels.

II. Article: Functional cross-modulation between SOCS proteins can stimulate cytokine signalling.

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III. References

Functional Cross-modulation between SOCS Proteins Can Stimulate Cytokine Signaling*

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SOCS proteins are regulatory elements that control cytokine signaling. Interactions between SOCS proteins and other signaling molecules are important for regulating cytokine signaling. In this study, we have investigated the role of SOCS2 in regulating cytokine signaling. SOCS2 is a negative regulator of cytokine signaling and its expression is regulated by cytokines. Our findings suggest that SOCS2 can interact with other SOCS proteins and that this interaction can modulate cytokine signaling. We also provide evidence that SOCS2 can interact with elongin BC complex, which is involved in protein degradation. These results highlight the complexity of the SOCS family and their role in regulating cytokine signaling.
SOCS Cross-regulation

come the negative effect of SOCS1 on GH receptor (GHR) and PRL signaling and can partially restore the SOCS3 down-regulated PRL function (26, 28, 30). Of note, SOCS6 overexpression also confers an enhanced phenotype because SOCS6 transgenic mice display increased insulin sensitivity and enhanced glucose metabolism (31).

GH, PRL, IFN, and others induce SOCS2 expression (27, 28, 32). Unlike SOCS1 and SOCS3 expression, which is typically induced in a rapid and transient manner, SOCS2 expression usually occurs later after cytokine stimulation and is more prolonged (28, 32). Consequently, it is tempting to speculate that SOCS2 may be involved in restoring cellular sensitivity by overcoming the inhibitory effect of other SOCS proteins. However, to date, no report concerning the precise molecular mechanism of action of SOCS2 in signal enhancement of GH response has been published.

This study was conducted to clarify the stimulatory effect of SOCS2 observed in GH signaling. We demonstrate that SOCS2 can interfere with the negative regulatory effects of SOCS1 and SOCS3 via direct interaction. This effect requires the C-terminal SOCS box of the targeted SOCS protein as well as the elongin BC-binding motif in the SOCS2 SOCS box, supporting proteasomal degradation of the targeted SOCS proteins. We also show that this inter-SOCS cross-modulation can be extended to other cytokine receptor systems and to other members of the SOCS protein family.

EXPERIMENTAL PROCEDURES

Constructs—All constructs used in this study were generated by standard PCR- or restriction-based cloning procedures and are represented in Table 1. The pEF-FLAG-I/mSOCS1, pEF-FLAG-I/mCIS, and pEF-FLAG-I/mSOCS2 constructs were kindly provided by Dr. R. Starr. pMET7-mLR (mouse leptin receptor (LR) long form) was a gift from Dr. L. Tartaglia, and the pc6b-rbGHR vector was a gift from Dr. G. Strous. The mouse thymus cDNA was kindly provided by Dr. P. Brouckaert. The pMET7-FLAG rat SOCS3 expression vector was described elsewhere (33). The pMET7-FLAG-CIS, pMET7-Etag-CIS, and pMET7-FLAG rat SOCS2 expression vectors have been described previously (34). Generation of the chimeric bait receptors containing the extracellular part of the erythropoietin receptor (EpoR) and the transmembrane and intracellular parts of the LR, such as pCEL, was described elsewhere (35, 36). Generation of the prey constructs pMG2-CIS and pMG2-SOCS2, both containing part of the gp130 chain (amino acids 905–918) in duplicate, was as described (37). The EpoR Tyr 402 double mutant and pMET7-SVT (SV40 large T antigen) expression vectors were obtained as described previously (36).

Cell Culture, Transfection Procedures, and Reagents—HEK293-T, 3T3-F442A, and N38 cells were cultured in a 10% CO2 humidified atmosphere at 37 °C and grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum (Cambrex Corp.). For transfection experiments, cells were freshly seeded in 6-well plates. HEK293-T cells were 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 load of the transcripitional and translational machinery. N38 cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s guidelines. One day after transfection, cells were washed with phosphate-buffered saline without calcium, magnesium, and sodium bicarbonate and cultured until used. Recombinant mouse leptin and human erythropoietin were purchased from R&D Systems. Human GH was purchased from ImmunoTools, and human IFN-β was generated in the laboratory.

Luciferase and Secreted Alkaline Phosphatase (SEAP) Assays—For a typical luciferase experiment, HEK293-T or N38 cells were transfected with the desired constructs together with a luciferase reporter gene. For STAT5-dependent luciferase assays, we used a STAT5-responsive β-casein-derived luciferase reporter plasmid (38). For STAT3-dependent luciferase experiments, we used the pXP2d2-rPAP1-luciferase reporter, originating from the rat pancreatitis-associated protein 1 promoter, as described previously (36). 24 h after transfection, cells were left untreated or were stimulated with ligand. After another 24 h, luciferase activity from triplicate samples was measured by chemiluminescence in a TopCount luminometer (Canberra Packard). For IFN-stimulated gene factor 3-dependent SEAP assays, we used the 6-16 SEAP reporter construct, which was constructed as described previously (39). The amount of SEAP was determined with a Phospa-Light kit (Tropix, Inc., Bedford, MA) using disodium 3-(4-methoxyisopropyl-1,2-dioxetane-3,2’-‘(5’-chloro)tricyclonenedecan-4-yl)phenyl phosphate as the luminogen substrate. Assays were performed in a 96-well microtiter plate following the manufacturer’s guidelines. Cells were lysed in 1% Triton X-100 and 20 mm Tris (pH 7.4), and alkaline phosphatase activity in triplicate samples was measured by chemiluminescence in the TopCount luminometer.

Western Blot Analysis and Co-immunoprecipitation—Transfected HEK293-T or N38 cells were lysed in modified radiomune precipitation assay buffer (200 mm NaCl, 50 mm Tris-HCl (pH 8), 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). 5× loading buffer (156 mm Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromphenol blue sodium salt, and 5% β-mercaptoethanol) was added to the cell lysates, which were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Blotting efficiency was checked by Ponceau S staining (Sigma). Blocking, washing, and incubation with antibodies were carried out in Tris-buffered saline supplemented with 5% dried skimmed milk and 0.1% Tween 20. FLAG-tagged (corresponding to the peptide tag DYYKDDDDK) and E-tagged (corresponding to the peptide tag GAPVPYDPLEPR) proteins were revealed using anti-FLAG monoclonal antibody M2 (Sigma) and anti-E tag monoclonal antibody (Amersham Biosciences), respectively. Rabbit anti-SOCS2 polyclonal antibody was a gift from Dr. J. Johnston, and anti-mouse β-actin antibody was supplied by Sigma. Immunoblots were then revealed by incubation with horsedavidianpheroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences) and SuperSignal West Pico chemiluminescent substrate (Pierce). For co-immunoprecipitation experiments, ~2 × 106 HEK293-T cells were transfected...
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with FLAG- or E-tagged pMET7-SOCS expression vectors. Cleared lysates (modified radioimmunoprecipitation assay lysis buffer) were incubated with 4.0 μg/ml mouse anti-FLAG or anti-E tag monoclonal antibody and protein G-Sepharose (Amersham Biosciences). After immunoprecipitation, SDS-PAGE, and Western blotting, interactions were detected using anti-FLAG or anti-E tag antibody as described above.

RESULTS

Essential Role for the SOCS2 SOCS Box in Antagonizing SOCS1 and SOCS3 Inhibition of Cytokine Signaling—SOCS2 exerts a dual action on GH and PRL signaling and impairs the inhibitory effect of other SOCS proteins (26, 30). To gain more detailed insight in the underlying mechanism, we first analyzed the role of the different SOCS protein subdomains. The effect of constitutive expression of SOCS proteins on GH signaling was investigated in HEK293-T cells using the STAT5-responsive β-casein-derived luciferase reporter. Fig. 1 shows dose-response curves demonstrating complete inhibition of GH signaling by SOCS1 and SOCS3. GH-inducible activity was fully inhibited by low concentrations of either SOCS1 or SOCS3, i.e. concentrations below the level of antibody detection as judged by Western blot analysis of the FLAG-tagged SOCS constructs using anti-FLAG antibody. After removal of the SOCS boxes of SOCS1 (SOCS1Δbox) and SOCS3 (SOCS3Δbox), inhibition was slightly reduced but not abolished in this assay system. Coexpression of SOCS2 completely suppressed the SOCS1- and SOCS3-dependent inhibition of GH signaling (Fig. 1, B and C), whereby SOCS1 inhibition appeared to be more sensitive to the counteracting effect of SOCS2 compared with SOCS3. Of note, the amounts of SOCS1 used in this experiment could not be visualized by Western blot analysis, indicating that the working concentrations

FIGURE 1. Essential role for the SOCS box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of GH signaling. HEK293-T cells were transfected with a rabbit GHR expression vector (40 ng) and a β-casein-derived luciferase reporter gene (200 ng). 24 h after transfection, the cells were deprived of serum and then treated with human GH (200 ng/ml) for 15 h before the luciferase activity from the β-casein reporter gene was measured. Luciferase measurements were performed in triplicate. Fold induction represents the ratio of luciferase activity determined in the presence or absence of ligand. A, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids (SOCS1Δbox (S1Δbox) and SOCS3Δbox (S3Δbox)) were cotransfected at a range of concentrations to analyze the inhibitory effect on GH signaling. A sample of lysate from each group was Western-blotted and probed with anti-FLAG antibody. B, SOCS1 (10 ng) or SOCS1Δbox (60 ng) was cotransfected with SOCS2 or SOCS2Δbox (52Δbox) at increasing concentrations. Expression of the E-tagged SOCS2 and SOCS2Δbox proteins in the same transfected cells was verified on lysates using anti-E tag antibody. C, SOCS3 (100 ng) or SOCS3Δbox (100 ng) was cotransfected with SOCS2 or SOCS2Δbox at increasing concentrations. Expression of the E-tagged SOCS2 and SOCS2Δbox proteins in the same transfected cells was verified on lysates using anti-E tag antibody. D, shows a comparison of the ectopic and endogenous expression levels of mouse SOCS2 in HEK293-T and 3T3-F442A cells, respectively. SOCS1 (10 ng) was cotransfected with SOCS2 at increasing concentrations in HEK293-T cells. Expression of the E-tagged SOCS2 proteins in the same transfected cells and of endogenous SOCS2 in the 3T3-F442A cells was verified on lysates using anti-SOCS2 antibody. 3T3-F442A cells were incubated in serum-free medium prior to stimulation with GH (200 ng/ml) for the indicated times. The levels of loaded protein were normalized by determining the protein concentrations with the Bradford method, and this was verified by Ponceau S staining. As an additional control, the blots were stripped and probed with anti-β-actin antibody to check for equal loading of lysates of the same cell type.
were approaching the physiological concentrations of this SOCS protein. The SOCS2 amounts were also not supra-physiological, as the SOCS2 concentration at which a cross-regulatory effect was observed was comparable with the endogenous level of SOCS2 in the GH-responsive mouse 3T3-F442A preadipocyte cell line (Fig. 1D). This suppressive effect on SOCS molecules was specific for SOCS2, as coexpression of CIS did not interfere with SOCS1- or SOCS3-mediated inhibition (data not shown). Strikingly, this effect of SOCS2 depended strictly on the presence of its SOCS box. Of note, the deletion of the SOCS box led to enhanced expression in the case of SOCS2. This effect was also observed, albeit to a lesser extent, with SOCS1, but was not observed for SOCS3.

We next evaluated whether we could extrapolate this SOCS2 regulation to other receptor systems. SOCS1 and SOCS3 have been implicated as potent inhibitors of IFN type I receptor signaling (40, 41); however, the role of SOCS2 is less well elucidated. We monitored IFN-β signaling in HEK293-T cells using the IFN-sensitive 6-16 SEAP type I reporter gene and evaluated the effect of expression of various combinations of (mutant) SOCS proteins as described above. We found that expression of SOCS2 at increasing concentrations resulted in a clear dual effect on IFN signaling (Fig. 2A): at low concentrations, SOCS2 suppressed IFN signaling, but at higher concentrations, SOCS2 led to complete restoration and even enhancement of the responsiveness of the 6-16 reporter to IFN-β, suggesting a negative effect of SOCS2 on endogenous SOCS proteins. Quite similar to what we observed with GH, expression of SOCS1, SOCS3, or their mutants lacking the SOCS box inhibited IFN-β signaling (Fig. 2B). Again, analogous to the observations made for GH, SOCS1- and SOCS3-mediated inhibition of IFN-β signaling was completely neutralized by coexpression of SOCS2, and the SOCS boxes of SOCS1 or SOCS3 and of SOCS2 were strictly required for the full effect (Fig. 2, C and D).

We finally extended these analyses of SOCS modulation also to LR signaling. Again, quite similar to the previous observations, expression of SOCS1, SOCS3, or their SOCS box deletion

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**FIGURE 2. Essential role for the SOCS box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of IFN signaling.** SEAP activity was assayed in HEK293-T cells transfected without SOCS or with SOCS1–3 expression vectors and the IFN-responsive 6-16 SEAP reporter (200 ng) at several ratios. After 24 h, transfected cells were stimulated with human IFN-β (100 pM); and after 48 h, the SEAP activity in the 6-16 reporter gene was measured. SEAP measurements were performed in triplicate. -Fold induction represents the ratio of SEAP activity determined in the presence or absence of ligand. A, SOCS2 can act as a dual effector of INF type I signaling as assessed by transfection with SOCS2 at a range of concentrations or with empty vector in an appropriate amount in HEK293-T cells. Expression of the E-tagged SOCS2 protein in the same transfected cells was verified on lysates using anti-E tag antibody. B, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were transfected at a range of concentrations to analyze the inhibitory effect on interferon type I signaling. C, SOCS1 (10 ng) or SOCS1Δbox (S1Δbox; 60 ng) was cotransfected with SOCS2 or SOCS2Δbox (S2Δbox) at increasing concentrations. D, SOCS3 (20 ng) or SOCS3Δbox (S3Δbox; 100 ng) was cotransfected with SOCS2 or SOCS2Δbox at increasing concentrations.
mutants blocked induction of leptin-mediated activation of a STAT3-responsive rat pancreatitis-associated protein 1 promoter-luciferase reporter in HEK293-T cells (data not shown). Coexpression of SOCS2 restored the SOCS-dependent signaling blockade. These effects were dependent on either SOCS box and were less pronounced for SOCS3-mediated LR inhibition than for SOCS1-mediated LR inhibition (data not shown). We further verified the cross-modulatory effects of SOCS2 in mouse N38 hypothalamic cells, which represent a physiological context for LR signaling. This N38 cell line responds to leptin stimulation and is a part of a collection of clonal neuronal cell lines recently isolated by Belsham et al. (42). Similar to what we observed in HEK293-T cells, expression of SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were cotransfected at a range of concentrations to analyze the inhibitory effect on leptin signaling. A, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were cotransfected at a range of concentrations to analyze the inhibitory effect on leptin signaling. B, SOCS1 (30 ng) or SOCS1Δbox (51Δbox; 100 ng) was cotransfected with SOCS2 or SOCS2Δbox (52Δbox) at increasing concentrations. C, SOCS2 or the SOCS2Δbox mutant plasmid was transfected at a range of concentrations to analyze the stimulatory effect on leptin signaling.

FIGURE 3. SOCS2 displays a SOCS box-dependent stimulatory effect on leptin signaling in N38 cells. Mouse N38 hypothalamic cells were transiently cotransfected with a mouse LR expression vector (250 ng) and the pXPF2d2-rPAP1-luciferase reporter (1 μg). The transfected cells were either left untreated or stimulated for 24 h with leptin (100 ng/ml). Luciferase measurements were performed in triplicate. Data are expressed as the leptin-stimulated/non-stimulated ratio. A, SOCS1, SOCS3, or their SOCS box deletion mutant plasmids were cotransfected at a range of concentrations to analyze the inhibitory effect on leptin signaling. B, SOCS1 (30 ng) or SOCS1Δbox (51Δbox; 100 ng) was cotransfected with SOCS2 or SOCS2Δbox (52Δbox) at increasing concentrations. C, SOCS2 or the SOCS2Δbox mutant plasmid was transfected at a range of concentrations to analyze the stimulatory effect on leptin signaling.

Recruitment of the Elongin BC Complex by SOCS2 Is Essential for Interference with Other SOCS Proteins—Sequence alignments of SOCS box-containing proteins reveal a single conserved region with the consensus sequence (T/S)(L/M)XXX(C/S)-XXX(V/L/I) that defines an elongin BC complex-binding site or “BC box” (9, 10, 43). We generated a SOCS2 mutant, SOCS2(LC-PF), containing point mutations in the BC box of SOCS2 that abrogate elongin BC recruitment (9). In another SOCS2 derivative, SOCS2(LC-QQ), both residues were mutated to glutamine to minimize structural alterations of the protein. As shown in Fig. 4, this SOCS2(LC-QQ) mutant completely lost its capacity to interfere with SOCS1 and SOCS3 antagonism in GH and IFN signaling in HEK293-T cells and with leptin signaling in N38 cells. Similar findings were made with the SOCS2(LC-PF) mutant (data not shown). This indicates that functional recruitment of the elongin BC complex is a prerequisite for the negative regulation by SOCS2 of other SOCS proteins.

SOCS2 Interacts with Other Members of the SOCS Family—We next used mammalian protein-protein interaction trap (MAPPIT), a strategy designed to analyze protein-protein interactions in intact mammalian cells (36), to investigate whether SOCS2 exerts its cross-modulatory function via direct binding to other SOCS proteins. In MAPPIT, a bait protein is C-terminally linked to a chimeric receptor consisting of the extracellular region of the EpoR linked to the transmembrane and the intracellular part of a signaling-deficient LR. The use of a triple Tyr-to-Phe mutant LR (further referred to as LR-F3) knocks out STAT3 activation and offers the added advantage that negative feedback mechanisms are inoperative, implying enhanced signaling.

MAPPIT prey constructs are composed of a prey protein fused to a part of the gp130 chain carrying four STAT3 recruitment sites. Coexpression of interacting bait and prey

SOCS Cross-regulation

SOCS2 displays a SOCS box-dependent stimulatory effect on leptin signaling in N38 cells.

The STAT3-dependent luciferase response (Fig. 3C), which can be explained by a negative effect of SOCS2 on the endogenous SOCS proteins. This effect was again lost with a SOCS2 mutant lacking the SOCS box. Together, these findings show that the cross-modulatory effect of SOCS2 on other SOCS proteins is not limited to the GH system and likely involves similar underlying mechanisms.

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Recruitment of the Elongin BC Complex by SOCS2 Is Essential for Interference with Other SOCS Proteins—Sequence alignments of SOCS box-containing proteins reveal a single conserved region with the consensus sequence (T/S)(L/M)XXX(C/S)-XXX(V/L/I) that defines an elongin BC complex-binding site or “BC box” (9, 10, 43). We generated a SOCS2 mutant, SOCS2(LC-PF), containing point mutations in the BC box of SOCS2 that abrogate elongin BC recruitment (9). In another SOCS2 derivative, SOCS2(LC-QQ), both residues were mutated to glutamine to minimize structural alterations of the protein. As shown in Fig. 4, this SOCS2(LC-QQ) mutant completely lost its capacity to interfere with SOCS1 and SOCS3 antagonism in GH and IFN signaling in HEK293-T cells and with leptin signaling in N38 cells. Similar findings were made with the SOCS2(LC-PF) mutant (data not shown). This indicates that functional recruitment of the
leads to functional complementation of STAT3 activity and induction of the STAT3-responsive rat pancreatitis-associated protein 1 promoter-luciferase reporter. MAPPIT permits the detection of both modification-independent and phosphorylation-dependent interactions in intact human cells. The MAPPIT configuration used in this study is shown in Fig. 5A.

We have shown previously that SOCS2 directly interacts with CIS (34). This observation and the abovementioned findings on cross-regulation between SOCS2 and SOCS1 or SOCS3 prompted us to investigate whether SOCS2 can bind to these SOCS family members. HEK293-T cells were cotransfected with a bait plasmid encoding the SOCS boxes of SOCS1, SOCS2, SOCS3, or CIS combined with a plasmid encoding the SOCS2 or CIS prey and the STAT3-responsive luciferase reporter construct. We always used isolated SOCS boxes as bait proteins because, in the case of SOCS1 and SOCS3, the full-length baits interfered with the MAPPIT readout and therefore could not be investigated. Erythropoietin stimulation revealed clear interaction of SOCS2 with all baits examined. In contrast, the CIS prey failed to induce any reporter activity (Fig. 5B). The expression levels of the FLAG-tagged prey proteins were confirmed by immunoblotting using anti-FLAG antibody. In Fig. 5C, we show, using MAPPIT, that the SOCS2(LC-QQ) mutant lost its capacity to associate with an elongin B prey while maintaining its interaction with the CIS bait. A MAPPIT bait construct containing the Tyr402 motif of the EpoR was used as a positive control, as this receptor motif directly interacts with SOCS2 (36). This association of SOCS2 with SOCS1–3 and CIS was confirmed by co-immunoprecipitation. We transiently cotransfected HEK293-T cells with a plasmid encoding E-tagged SOCS2 together with FLAG-tagged plasmids encoding SOCS1, SOCS2, and SOCS3, respectively, or we coexpressed FLAG-tagged SOCS2 with E-tagged SOCS1, SOCS2, and CIS. SOCS2 co-immunoprecipitated SOCS1–3 (Fig. 6A) and, in the case of CIS, both the 37- and 32-kDa forms that correspond to monoubiquitinated and non-ubiquitinated proteins, respectively (44). Observed interactions of SOCS2 with SOCS proteins in HEK293-T cells depended on proteasomal inhibition with the proteasomal inhibitor MG132 (20 μM) for 6 h and stimulation with IFN-β (100 μM) for 30 min. SOCS1 was still co-immunoprecipitated with the SOCS2(LC-QQ) mutants, indicating that deletion of the SOCS box or the BC box motif did not disrupt the capacity of SOCS2 to bind SOCS1 (Fig. 6B). Nevertheless, elimination of the SOCS box of SOCS2 weakened the interaction with SOCS1, suggesting a role for this domain in SOCS-SOCS interactions.
SOCS Cross-regulation

A. [Diagram showing protein interactions and signaling pathways related to SOCS regulation]

B. [Graphs showing fold induction for different conditions]

C. [Graphs showing fold induction for different conditions]
SOCS2 Promotes Degradation of SOCS1—The dependence of the SOCS2 effect on an intact BC box suggests that SOCS2 can target SOCS proteins for proteasomal degradation. HEK293-T cells were transiently transfected with SOCS1 and increasing concentrations of SOCS2 and then treated with the protein synthesis inhibitor cycloheximide (20 μM) for 6 h. Degradation of SOCS1 was observed when increasing concentrations of SOCS2 were coexpressed, whereas SOCS2(LC-QQ) had no effect (Fig. 7). This suggests a mechanism in which SOCS2 acts as an adaptor molecule between an E3 complex and SOCS proteins, targeting them for proteasomal turnover.

MAPPIT interaction had no deleterious effect (data not shown). From these experiments, we concluded that SOCS2, SOCS6, and SOCS7 can interact with the SOCS boxes of all SOCS family members.

SOCS6 Is a Negative Regulator of Other SOCS Proteins—Subsequently, we investigated whether SOCS6 displays similar functional SOCS cross-modulation as SOCS2. We found that SOCS6 antagonized the inhibition of SOCS proteins in GH and IFN (Fig. 9, A and B) and leptin (data not shown) signaling in HEK293-T cells. In N38 cells, SOCS6 interfered with SOCS1 and SOCS3 inhibition of leptin signaling (Fig. 9C).

SOCS2 and Also SOCS6 and SOCS7 Interact with All Members of the SOCS Family—Interaction studies performed with the other SOCS proteins used as baits revealed that the SOCS2 prey can also interact with the SOCS boxes of SOCS4–7 (Fig. 8A). Using the same approach, we performed a matrix-type interaction analysis of SOCS proteins, and we found that SOCS6 and SOCS7 preys also interacted with the SOCS box baits of all members of the SOCS family (Fig. 8, B and C). Of note, SOCS2, SOCS6, and SOCS7 also displayed binding to themselves. The MAPPIT data with the CIS prey were included as a negative control, and functionality of this CIS prey was demonstrated using the interaction with the EpoR Tyr402 motif as a control (Fig. 8D). The EpoR-LR-F3 bait provided a control for aspecific binding to the intracellular part of the LR and JAK2. The expression of the different bait constructs was verified by checking the interaction with the SH2β prey, which binds the associated JAK of LR-F3 (data not shown). Possible complications that could arise from interference of the SOCS prey constructs with JAK activity or STAT recruitment and that could lead to false negative signals were considered and ruled out, as their coexpression with an established

**FIGURE 5.** MAPPIT analysis of SOCS interactions. A, principle of MAPPIT. See “Results” for details. pRPA1, rat pancreaticis-associated protein 1. B, SOCS2 interacts with the SOCS boxes of SOCS1–3 and CIS. HEK293-T cells were transiently cotransfected with plasmids encoding bait variants of the SOCS boxes of several SOCS proteins or with a mock bait lacking the SOCS motif, the pMG2-SOCS2 or pMG2-CIS prey construct, and the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with erythropoietin (Epo) for 24 h. Luciferase activities were measured in triplicate. Fold induction represents the ratio of luciferase activity determined in the presence or absence of ligand. Expression of the FLAG-tagged fusion prey proteins in the same transfected cells was verified on lysates using anti-FLAG antibody. C, the SOCS2(LC-QQ) mutant does not bind elongin B, whereas the interaction with CIS is preserved. HEK293-T cells were transiently cotransfected with plasmids encoding the chimeric EpoR-LR-F3 construct as a negative control, the EpoR Tyr402 bait as a positive control for SOCS2, the elongin B bait, or the CIS SOCS box bait and with the pMG2-SOCS2 or pMG2-SOCS2(LC-QQ) prey construct combined with pXP2d2-rPAP1-luciferase. The transfected cells were either stimulated for 24 h with erythropoietin or were left untreated. Luciferase measurements were performed in triplicate. Data are expressed as fold induction (stimulated/non-stimulated).

**FIGURE 6.** A, SOCS2 interactions demonstrated by co-immunoprecipitation experiments. Lysates from HEK293-T cells cotransfected with FLAG- or E-tagged SOCS1 (S1), SOCS2 (S2), SOCS3 (S3), and CIS were immunoprecipitated (IP) with anti-E tag or anti-FLAG antibody and Western-blotted (WB) with anti-FLAG or anti-E tag antibody (upper panels). The whole cell lysate was Western-blotted with anti-FLAG or anti-E tag antibody as a loading control (middle and lower panels). B, interaction analysis of SOCS1 and SOCS2 mutants. HEK293-T cells were transiently cotransfected with FLAG-tagged SOCS2, SOCS2Δbox (S2Δbox), SOCS2ΔBC box(LC-QQ) (S2(LC-QQ)), or the appropriate amount of empty vector and E-tagged SOCS1. Cell lysates were immunoprecipitated with anti-FLAG antibody and subsequently immunoblotted with anti-E tag or anti-FLAG antibody.
Like other SOCS molecules, SOCS6 was shown to bind to elongins B and C in a SOCS box-dependent manner (45). Analogous to SOCS2, disruption of elongin BC binding in SOCS6 yielded a mutant that was not able to interfere with the inhibitory effect of other SOCS proteins (Fig. 9). Also, wild-type SOCS6, but not the ΔBC box mutant, reduced SOCS1 expression in a dose-dependent manner, indicating that SOCS6 mediated the observed inhibition by accelerating turnover of other SOCS proteins (Fig. 10). Taken together, our data suggest that SOCS6 can negatively regulate SOCS function in a way very similar to SOCS2.

**DISCUSSION**

Protein degradation by the ubiquitin-proteasome pathway plays an essential role in controlling the abundance of regulatory molecules. Key to this is the sequential action of three protein sets: ubiquitin-activating enzymes, ubiquitin carrier enzymes, and a large set of E3 enzymes, whereby the latter define substrate specificity. The SCF (Skp1-Cul1-F-box) E3 complex is composed of the Cul1 scaffold protein, which binds the Roc1/Rbx1 RING domain protein and the ubiquitin carrier enzyme and which recruits, via the Skp1 linker protein, F-box proteins, which in turn bind substrates for ubiquitination. This same architecture is also found in other SCF-like complexes, including those based on the Cul2-von Hippel-Lindau and Cul5-SOCS box adaptor proteins, whereby elongins B and C and SOCS or von Hippel-Lindau proteins fulfill the role of the Skp1 and F-box protein moieties, respectively (1). Evidence that SOCS proteins can mediate proteasomal turnover of target molecules is accumulating. Examples include the GHR and EpoR (44, 46), JAK2 (13), the Rac guanine nucleotide exchange factor Vav (47), Ras GTPase-activating protein (17), and insulin receptor substrates 1 and 2 (48). Of note, SOCS proteins themselves can be targeted for ubiquitination and proteasomal degradation, although contradictory reports exist regarding the effect of elongin BC interaction on the protein stability of SOCS1, SOCS3 and CIS. Some data suggest that elongin BC association targets SOCS proteins for degradation by the proteasome, as has been demonstrated for CIS (44, 49), SOCS1 (10, 50), and SOCS3 (10, 51). In contrast, there is also evidence that elongin BC interaction can stabilize SOCS1 (9, 16, 52) and SOCS3 (17) and that disruption of this interaction leads to proteasome-mediated degradation of these SOCS proteins.

SOCS2 undeniably plays a role as a negative regulator of GH signaling in vivo and in vitro (43), but can also enhance GH signaling when expressed at higher concentrations (25, 26). It binds to the GHR at multiple sites, some of which could also function as recruitment sites for negative regulators such as SHP-2 (53) and SOCS3 (54, 55). Such competition between SOCS2 and potentially more potent negative regulators was put forward as a potential explanation for the dual effect of SOCS2 (25). However, little direct evidence was reported in support of such a model, and recently, Greenhalgh et al. (43) showed that SOCS2 binds the GHR at Tyr^{487} and Tyr^{595}, which are not usual immunoreceptor tyrosine-based inhibitory motifs, suggesting that competition of SOCS3 at these sites is not involved.

The key finding in this study is that a restricted set of SOCS proteins, including SOCS2, can bind to other members of the SOCS family, thus controlling their activity through proteasome-dependent degradation. We found that SOCS2 can restore and potentiate GH signaling by antagonizing SOCS1 and SOCS3 in a SOCS box-dependent manner. This effect is not limited to the GH system because we found similar effects on signaling via the endogenous IFN type I receptor and LR in HEK293-T and N38 cells, respectively.

SOCS2 mutants lacking the binding site for elongin BC completely lose their inhibitory potential, providing a strong argument for proteasomal degradation of the target SOCS proteins. Indeed, as observed for SOCS1, coexpression of SOCS2 leads to lowered expression levels of this target SOCS protein. The critical elongin BC dependence of the inhibitory effect by SOCS2 strongly argues that SOCS2 functions as part of an E3 complex. Alternatively, higher expression levels of SOCS2 may compete for recruitment of the elongin BC complex, indirectly leading to destabilization of other SOCS proteins lacking this complex (see above). However, SOCS1 and SOCS3 proteins lacking their entire SOCS boxes are still able, although to a lesser extent, to inhibit cytokine signaling, but are completely refractory to the SOCS2 effect, implying that SOCS2 binding is critical. Moreover, overexpression of CIS, which is equally well capable of sequestering elongin BC complexes, does not lead to any effect on other SOCS proteins. The SOCS box of the target SOCS protein appears to be involved in SOCS2 binding. Although our data support an involvement of the SOCS box in the interaction between the inhibitory SOCS and targeted SOCS proteins, the precise nature of this inter-SOCS interaction is still unclear and, given the MAPPIT configuration, may well depend on phosphorylation of critical tyrosine residues. Mutational analysis will be required to fully determine the binding modus between different SOCS proteins.

Evidence that SOCS2 can act as a regulator of turnover of other SOCS proteins was recently also reported by Tannahil et al. (29), who demonstrated SOCS2 regulation of the SOCS3-dependent inhibition of interleukin-2 and interleukin-3 signaling, and by Lavens et al. (34), who showed elongin BC-dependent interference of SOCS2 with binding of CIS to the LR at Tyr^{985}. In line with such a regulatory role of SOCS proteins is the sequential induction pattern of different SOCS molecules. Unlike expression of SOCS1 and SOCS3, which is typically induced in a rapid and transient manner upon cytokine stimulation, expression of SOCS2 usually occurs late after cytokine...
FIGURE 8. SOCS2, SOCS6, and SOCS7 interact with the SOCS boxes of all SOCS family members. HEK293-T cells were transiently cotransfected with plasmids encoding bait variants of the SOCS boxes of all SOCS proteins or the chimeric EpoR-LR-F3 construct as a negative control; the pMG2-SOCS2, pMG2-SOCS6, pMG2-SOCS7, or pMG2-CIS prey; and the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with erythropoietin (Epo) for 24 h. Luciferase activities were measured in triplicate. Data are expressed as -fold induction (stimulated/non-stimulated).
FIGURE 9. The negative effect of SOCS6 on other SOCS proteins depends on recruitment of the elongin BC complex. HEK293-T or N38 cells were transiently transfected with plasmids encoding SOCS1 or SOCS3 at fixed amounts and wild-type SOCS6 or SOCS6ΔBC box(LC-QQ) (S6Δ(LC-QQ)) at increasing concentrations. The transfected cells were either left untreated or were stimulated with GH (200 ng/ml), IFN-β (100 pM), or leptin (100 ng/ml). Data are expressed as fold induction (stimulated/non-stimulated). Luciferase and SEAP measurements were performed in triplicate. A, HEK293-T cells were transfected with 10 ng of SOCS1, 100 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. GH signaling was assayed as described in the legend to Fig. 1. B, HEK293-T cells were transfected with 10 ng of SOCS1, 50 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. IFN signaling was assayed as described in the legend to Fig. 2. C, N38 cells were transfected with 30 ng of SOCS1, 10 ng of SOCS3, and increasing concentrations of SOCS6 derivatives. Leptin signaling was assayed as described in the legend to Fig. 3.
stimulation and is more prolonged (27, 28, 32). Accumulation of increasing levels of SOCS2 late after induction is consistent with a role in eliminating excess levels of SOCS proteins after receptor activation and may be involved in restoring cellular responsiveness for subsequent stimulation. Interestingly, SOCS2, SOCS6, and SOCS7 can also bind to themselves, suggesting the possibility of self-elimination. A full and global insight into the precise inhibitory effects will thus require careful full analysis of the interaction pattern at the cytokine receptor, at the targeted SOCS protein, and at the level of self-interaction, bearing in mind the effect of the differences in binding affinities and relative expression levels of all components.

Whereas SOCS1–3 and CIS have been studied extensively, so far little is known about the physiological role of the other four SOCS proteins, SOCS4–7. We therefore analyzed matrix-wise all possible inter-SOCS interactions. The interaction map showed two characteristics: first, SOCS2 appears to bind to all SOCS proteins, including itself; and second, SOCS6 and SOCS7 display exactly the same binding profile.

In line with a SOCS-counteracting role of SOCS6, we have shown that its expression potentiates signaling via the GH, IFN type I receptor, and LR in a way quite similar to what we observed for SOCS2. Similar data sets were also obtained for leptin signaling in the physiologically relevant N38 hypothalamic cell line. These novel findings regarding SOCS6 provide an explanation for the significant enhancement of glucose metabolism observed in SOCS6 transgenic mice (31). More evidence for a positive role for SOCS6 in cytokine signaling also comes from studies in Drosophila melanogaster, where SOCS44A (which is similar to SOCS6) was shown to enhance the activity of the growth factor receptor/mitogen-activated protein kinase (MAPK) signaling cascade, in contrast to SOCS36E (which is similar to SOCS5) (56).

Because several SOCS molecules can mediate similar regulatory effects, functional redundancy is not unlikely. This may be particularly true for (but not limited to) SOCS6 and SOCS7, which show high homology and similarity in binding specificity (45). Compensatory effects between cross-modulatory SOCS molecules may perhaps have an effect on the phenotypes of SOCS2-, SOCS6-, and SOCS7-deficient mice (23, 45, 57), warranting analysis of double knock-out mice, which may uncover additional physiological activities of particular SOCS proteins. Of note, the SOCS box is not limited to the SOCS protein family, but at present, 128 proteins harboring a SOCS box have been described in the mammalian genome (according to the Pfam Database).

In summary, our findings point to the existence of a subfamily of SOCS proteins consisting of SOCS2, SOCS6, and SOCS7, capable of controlling SOCS protein stability. This functional cross-modulation between SOCS proteins requires the SOCS box, probably both as an inter-SOCS-binding domain and as a functional recruitment motif for elongin BC-containing E3 enzymes. The observation that several SOCS proteins do not act solely as inhibitors of cytokine signaling should be taken in consideration in the evaluation of gene knock-out studies and may be of relevance for several human pathologies.

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Note Added in Proof—After submission of this manuscript, a paper was published by Ouyang et al. (58) demonstrating positive effects of SOCS2 upon ectopic expression in C2C12 myoblasts.

REFERENCES


FIGURE 10. SOCS6, but not SOCS6 (LC-QQ), promotes degradation of SOCS1. Increasing concentrations of FLAG-tagged SOCS6 or SOCS6 (LC-QQ) were coexpressed transiently in HEK293-T cells with FLAG-tagged SOCS1. Cells were treated with cycloheximide (20 μM) for up to 6 h. The lysates were blotted for SOCS1 and SOCS6 expression with anti-FLAG antibody.
CHAPTER 10: CIS functions are controlled by Elongin B/C binding

I. Introduction
Elongin B/C association is known to be involved in proteasomal targeting of substrates and regulation of protein stability. In the following study we show that Elongin B/C recruitment to the SOCS box of a protein can also control its substrate interaction. Receptor interaction of CIS and subsequent inhibition of STAT5 activity were found to crucially depend on Elongin B/C binding. This Elongin B/C-dependency for substrate interaction appears to be unique for CIS and structural basis for this distinct feature of CIS is provided by molecular modelling of the CIS-Elongin B/C complex. These findings imply a new role for the SOCS box which may form a regulatory on/off switch acting on the SH2 domain. Increasing evidence indicates a key role for proteasomal activity in CIS-mediated signal suppression (Hunter et al., 2004; Ram and Waxman, 2000; Uyttendaele et al., 2007). Our observation that Elongin B/C association cannot be uncoupled from CIS actions further supports a major contribution for the recruitment of E3 ligase activity in CIS-mediated inhibition.

II. Article: Elongin B/C recruitment regulates substrate binding by CIS.
Submitted to Journal of Biological Chemistry, 2008

III. References
SOCS proteins play a major role in the regulation of cytokine signalling. They are recruited to activated receptors and can suppress signalling 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 here describe a novel regulatory mechanism for CIS, one of the members of this protein family. A CIS mutant that is deficient in recruiting the Elongin B/C complex completely fails 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 is easily disrupted and depends on the levels of uncomplexed Elongin B/C. This regulatory mechanism may be unique for CIS since 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 signalling events, including activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphoinositol 3-kinase (PI3K), phospholipase C \( \gamma \) (PLC\( \gamma \)) and mitogen activated protein kinase (MAPK) 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 protein tyrosine phosphatase 1B (PTP-1B) or T-cell protein tyrosine phosphatase (TCPTP) (1-5), members of the protein inhibitors of activated STATs (PIAS) (6-8) and suppressor of cytokine signalling (SOCS) 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 (12,13,14,15,16). 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 carboxy-terminal 40 amino-acid module that is known as the SOCS box (9,17,18). 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 (18-20). This way, SOCS proteins can suppress signalling by direct competition with signalling molecules for the phosphorylated recruitment sites. SOCS1 and SOCS3 can also inhibit JAK tyrosine kinase activity through their kinase inhibitory region (KIR), which is proposed to function as a pseudosubstrate blocking the catalytic cleft of the JAK kinase (21). Finally, SOCS proteins can suppress signalling through proteolytic degradation of the activated receptor complexes. Conserved motives in their SOCS box couple to Elongin B and C (B/C), Cullin and Rbx proteins, leading to the formation of an E3 ubiquitin ligase complex (22-25) and subsequent ubiquitin-marking of the target protein for proteasomal degradation. The functional significance of the association of Elongin B/C to the SOCS box is however complex as the SOCS box may also target SOCS proteins themselves for proteasomal degradation (23,26,27). Conversely, Elongin B/C binding was also found to stabilize SOCS
protein expression (22,28,29). 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 (30-32). Furthermore, we reported 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 (33).

The founding member of the family, CIS, can inhibit signalling 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 (34,35). CIS transgenic mice exhibit growth retardation, suggesting a defect in GH signal transduction (36). Other abnormalities of CIS overexpressing mice were detected in prolactin and IL-2 signalling pathways and these phenotypes resembled those found in STAT5a and/or STAT5b knock-out mice, lending support for CIS as a specific negative regulator of STAT5-mediated cytokine signalling (36). Direct competition with STAT5 for common phosphoryrosine binding sites is observed for the EpoR (37-40), but not for the GHR (41). In addition, CIS was found to induce proteasome-dependent degradation of the EpoR and GHR (37,42,43) and in the latter case, CIS also was reported to play a role in GHR internalization (43). In this report, we investigated the effect of Elongin B/C binding on CIS function in greater detail. Notably, we found that Elongin B/C recruitment by CIS is crucial for interaction with its receptor substrates and subsequent inhibition of STAT5 activity.

**Experimental Procedures**

**Constructs-** All constructs used in this study were generated by standard PCR- or restriction-based cloning procedures and are represented in table 1. The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was described previously (44). The pGL3-β-casein-luciferase reporter consisting of five repeats of the STAT5-responsive motif of the β-casein promoter was a gift from Dr. Ivo Touw (45) and the STAT5-responsive pGL2-SP12.1-luciferase reporter was a gift from Dr. Walter Becker (46). The pRK5-mJAK2 construct was a gift from Dr. Yohan Royer. Generation of the chimeric bait receptors containing the extracellular part of the EpoR and the transmembrane and intracellular parts of the leptin receptor, such as pCEL, were reported elsewhere (44,47). The following constructs were described previously: mLR Y1138F (YYF) (48); pCEL-EpoR Y402 and pMG-SVT (44); pCEL-GHR Y595 (41); pSV-EpoR pMG2-CIS and pMG2-SOCS2 (40); pMET7-FLAG-CIS and pMET7-FLAG-SOCS2 (49); pMG2-CISY253F and pCAGGS-E-mMyD88-DD (33); pMG2-SOCS2AB/C, pMG2-SOCS6, pMG2-SOCS7, pMET7-E-SOCS1 and pMET7-FLAG-SOCS2AB/C (31); pMET7-FLAG-SOCS3 (50).

**Cell culture, transfection procedures and reporter assay-** HEK293-T cells were cultured in 8% CO2 humidified atmosphere at 37°C, and grown using Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum (Cambrex Corp.). For transfection experiments, HEK293-T cells were freshly seeded in 6-wells plates and transfected overnight with approximately 2,5 µg plasmid DNA using a standard calcium phosphate precipitation procedure. The pMET7-SVT construct was used to normalize for the amount of transfected DNA and load of the transcriptional and translational machinery. The next day, cells were washed with phosphate-buffered saline (PBS), transferred to a 96-well plate and left untreated or stimulated with ligand for at least 24 h. Recombinant mouse leptin and human erythropoietin (Epo) 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/non-stimulated). 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 µF). 48 h after transfection cells were simultaneously starved (removal of serum and mIL-3) for 24 h and stimulated with 1 ng/ml mIL-3 overnight. Activation of the pGL2-SP12.1-luciferase reporter
was measured with the Topcount luminometer (Canberra Packard).

**Western blot analysis and co-immunoprecipitation**

Transfected HEK293-T cells were lysed in modified RIPA buffer (200 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.05% SDS, 2mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1mM Na₃VO₄, 1mM NaF, 20 mM β-glycerophosphate and Complete™ protease inhibitor cocktail (Roche Applied Science)). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C. 5x loading buffer (156 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol) was added to the cell lysates, which were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Blotting efficiency was checked by Ponceau S staining (Sigma). FLAG-tagged (corresponding to the peptide tag DYKDDDDK) and E-tagged (corresponding to the peptide tag GAPVPYPDPLEPR) proteins were revealed using respectively monoclonal anti-FLAG antibody M2 (Sigma) and monoclonal anti-E-tag antibody (GE Healthcare). Polyclonal rabbit anti-CIS, goat anti-Elongin C and rabbit anti-JAK2 were supplied by Santa Cruz Biotechnology. Western blot analysis was performed using the Odyssey Infrared Imaging System (Li-Cor) following the manufacturer’s guidelines. In brief, blots were blocked in Odyssey blocking buffer (Li-Cor), then probed overnight with the appropriated antibodies diluted in Odyssey blocking buffer with 0.1% Tween 20 and finally incubated with an IRDye 700 donkey anti-goat, IRDye 800 goat anti-mouse or IRDye 800 goat anti-rabbit secondary antibody (Li-Cor). For the co-immunoprecipitation experiments approximately 6 x 10⁶ HEK293-T cells were transfected with FLAG-tagged or E-tagged expression vectors using the calcium phosphate transfection procedure and lysed after 48 h in 1ml lysis buffer (50mM NaCl, 20mM Tris-HCl pH6.6, 1% Nonidet P-40, 2.5% glycerol, 1mM EDTA, 1mM Na₃VO₄, 1mM NaF, 20 mM β-glycerophosphate and Complete™ protease inhibitor cocktail (Roche Applied Science)). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C and incubated with 4,0 μg/ml anti-FLAG mouse monoclonal antibody (Sigma) or anti E-tag mouse monoclonal antibody (GE Healthcare) and protein G-sepharose (Amersham Biosciences). After immunoprecipitation and SDS-PAGE, interactions were detected by immunoblotting using the appropriate antibody.

**(Phospho)peptide affinity chromatography**

Approximately 2 x 10⁷ HEK293-T cells transiently transfected as indicated were lysed in 5 ml lysis buffer (150 mM NaCl, 20 mM HEPES pH 7.8, 0.5% Nonidet P-40, 20% glycerol, 1 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1 mM Na₃VO₄, 1 mM NaF, Complete™ Protease Inhibitor Cocktail (Roche Applied Science)). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C and loaded on a pre-column with Sepharose 4B beads and streptavidin-agarose to prevent nonspecific interactions. Pre-cleared lysates were then incubated for 2 h at 4°C with the (phospho)tyrosine peptides as indicated coupled to streptavidin-agarose beads through their biotin group. The beads were then washed twice with lysis buffer and resuspended in 2x loading buffer (62.5 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol). Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody. The sequences of the used peptides were biotin-EGASAASFEY(T)ILDPSSQL for Y⁴₀² of the EpoR and biotin-QRQPSVKY(T)ATLVSNDK for Y⁹₈₅ of the LR. Synthesis and purification of the biotinylated (phospho)tyrosine peptides and coupling to streptavidin-agarose beads was described before (50).

**Gel filtration chromatography**

Approximately 10⁸ Ba/F₃ cells were stimulated with 10 ng/ml IL-3 for 1h at 37 °C and lysed in 3 ml lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8, 2 mM EDTA, 0.875% Brij 97 (Sigma-Aldrich), 0.125% Nonidet P-40, 1mM Pefabloc and Protease Inhibitor Cocktail Set III (Calbiochem). Lysates were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C, quantified with the Bradford method and applied to a calibrated Superdex 75 PG16/60 gel filtration column (Amersham) run by an Advanced Protein Purification System (APPS) apparatus (Waters). The running buffer consisted of 50 mM Tris-HCl pH 7.4 and 150 mM NaCl. After trichloroaceticacid (TCA) precipitation of equal volumes of collected fractions, the samples were subjected to SDS-PAGE and immunoblotting using the indicated antibodies.
Modelling method- Homology models for CIS, based on the crystal structures of SOCS2 (51) or SOCS4 (52) were created as described before (41).

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: CISΔB/C (L222,Q,C228,Q) (22) and CISΔCul (L240PLP244-AAAA) (53) (Fig. 1A). CIS activity was evaluated by a STAT5-dependent reporter assay in HEK293-T cells that were transiently transfected with the human EpoR expression vector and CIS, SOCS2 or mutant constructs. While wild type CIS clearly suppressed reporter activity as expected, the CISΔB/C derivative was almost completely unable to impair reporter induction (Fig. 1B). In contrast, the CISΔCul variant was still fully functional, indicating that the effect of the CISΔB/C mutation was specifically due to abrogated Elongin B/C recruitment and not to a lack of formation of a larger E3 complex. As a control, we also included the Y253F loss-of-function CIS mutant (33). Much in contrast, a similar B/C box mutation in the highly related SOCS2 protein (SOCS2ΔB/C, L167Q,C167Q) showed no effect on STAT5 signalling. To examine the function of the CIS mutants in a more physiological set-up, STAT5-dependent reporter assays measuring interleukin-3 (IL-3) signalling 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 (18,20). In case of CIS, we recently demonstrated that the carboxy-terminal Y253 residue is also required for interaction with phosphotyrosine motifs in cytokine receptors (33). Since 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 analyse protein-protein interactions in mammalian cells. In MAPPIT, a bait protein is carboxy-terminally linked to a chimaeric receptor that is deficient in STAT3 recruitment, while 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. MAPPIT permits the detection of both modification-independent and phosphorylation-dependent interactions. The MAPPIT configurations used in this manuscript are described in Figure 2A. Functional expression of the different bait constructs was assessed by measuring interaction with the JAK2-binding Ring Finger Protein 41 (RNF41) prey (De Ceuninck et al., unpublished results). 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 Y1077 of the EpoR (44) and Y985 of the GHR (41) as baits. We also analysed interactions with the leptin receptor (LR) by mutating the STAT3-recruiting Y985 to F (LR (YYF)). This way, MAPPIT analysis of interactions at positions Y985 and Y1077, that are known to recruit SOCS proteins (49,50,54) 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 rPAP1 luciferase reporter. Figure 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 Y1077 bait were confirmed by phosphopeptide affinity chromatography. Complete loss of binding to the phosphorylated Y402 of the EpoR was observed for the CISΔB/C or Y253F mutants, while no effect was seen for a similar SOCS2ΔB/C mutant (Fig. 2C).

In analogy with the observed interaction pattern of the CIS Y253F mutant (33), deletion of the B/C box did not affect interaction with the TLR 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 Figure 2E we integrated binding controls for the CIS mutants. The top panel shows a MAPPIT experiment demonstrating that the CISΔB/C, but not the CISΔCul prey is incapable to interact with
an 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 CISΔB/C bait, whilst interaction of only the Cullin5 prey is lost for the CISΔCul mutation. Finally, the bottom panels show co-immunoprecipitation experiments demonstrating loss of interaction with the endogenous Elongin C and FLAG-tagged Cullin5 upon deletion of the B/C motif or the Cullin box in CIS.

Loss of substrate binding by CISΔB/C is not due to an effect on CIS stability. Although we did not observe a significant effect of the CISΔB/C mutation on protein expression, we wanted to verify whether an altered protein half-life time could account for the loss of substrate binding. To this end, protein expression levels of CIS (with or without co-expressed Elongin B/C) and of the CISΔB/C mutant were monitored in the presence of the translation inhibitor cycloheximide and half-lives were determined by quantification of the observed band intensities. As shown in Figure 3, the decline of the mutant was less pronounced than that of wild type CIS. The estimated half-life was 40' for CIS and 1 h 30' for CISΔB/C respectively, ruling out an effect of protein stability on the interaction assays. Of note, Elongin B/C co-expression also significantly extended CIS half-life time, indicating a stabilizing effect of Elongin B/C.

The CIS Elongin B/C-dependency for substrate interaction is unique among SOCS proteins. We next questioned whether other SOCS family members also display a similar dependency 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 ΔB/C mutation in SOCS6 or SOCS7 did not alter the interaction pattern (Fig. 4A). Other read-outs were used for SOCS1 and SOCS3 since these inhibit the MAPPIT assay due to their JAK suppressive activity. The interaction of SOCS1 with JAK2 was analysed by co-immunoprecipitation. As shown in Figure 4B, SOCS1 and its ΔB/C mutant bind equally well to JAK2. For SOCS3, no effect was seen for the SOCS3ΔAB/C mutant in a STAT5-dependent reporter assay implying normal interaction with the EpoR (Fig. 4C). Similarly, normal binding was seen for the SOCS3ΔAB/C mutant in a phosphopeptide affinity chromatography experiment using the phosphorylated Y985 motif of the LR (Fig. 4D).

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 set-up. 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 that 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, in comparison with SOCS2. 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 functionality is regulated by the intracellular level of free Elongin B/C complex, we performed two distinct experiments. First, we examined using MAPPIT whether co-expression of Elongin B/C in HEK293-T cells could enhance CIS substrate binding. As shown in Figure 6A, this is clearly the case using the GHR Y595 motif as bait, while Elongin B/C co-expression was not inducing 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. We therefore examined in a second experiment 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 Figure 6B the majority of CIS eluted in fractions corresponding to complexes of smaller molecular weight or monomeric CIS (< 37 kDa in mass) and only a subset of the total CIS pool co-eluted with Elongin
C (complex of > 44.6 kDa). 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 B/C and Cullin boxes respectively mediate Elongin C (22,55-58) and Cullin2 or 5 recruitment depending on the SOCS family member (53). We here report 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 solely binding of Elongin B/C, 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: partial inhibition by direct competition with STAT5 for common phosphoryrosine binding sites on the receptor and proteasome-mediated degradation of the receptor-JAK2 signalling complex. In case of the GHR, this latter mechanism may be coupled to internalization of the activated receptor complex, a critical step preceding termination of receptor signalling (43). SOCS-mediated inhibition of the GHR by competition for shared binding sites with STAT5 was recently ruled out based on the non-overlapping bindings pattern of CIS and SOCS2 with STAT5 (41). Furthermore, the SOCS box of CIS was found to be essential for the apoptotic effect of CIS on erythroid progenitor cells (35). 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 abolish completely the inhibitory functions of CIS in EpoR (and IL-3R, data not shown) signalling and 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. Since 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 carboxy-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 (33,41). 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 the Elongin B/C motif or Y253 in CIS both lead to loss of binding to all substrates that were tested, except MyD88. Together, these data suggest that the Elongin B/C mutation and Y253 mutation affect receptor binding through a common mechanism.

Two different homology models were built for CIS, with a SOCS box orientation as in SOCS2 (homology model 1, Fig. 7A) or SOCS4 (homology model 2, Fig. 7B). Both models exclude a direct interaction of the SOCS box or Y253 with the phosphopeptide substrate. In the model, Y253 shows no direct interaction with the Elongins and mutation of Y253 in CIS both lead to loss of binding to all substrates that were tested, except MyD88. Similar structural rearrangements take place in yeast Elongin C upon binding of a SOCS box peptide from the von Hippel-Lindau (VHL) protein (59). VHL requires Elongin B/C binding to adopt a stable structure (60,61) 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, since B/C box independent interactions of CIS
were still observed with MyD88. We verified whether the loss-of-function of the CISΔB/C mutant was not due to an altered half-life time. The particular CIS mutant appeared to be even more stable that 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.

In co-immunoprecipitation experiments CIS appeared to display a lower affinity for Elongin C than SOCS2. Furthermore, MAPPIT competition experiments showed that SOCS2 over-expression 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 later stage 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 previously reported that this involves the SOCS box of CIS as interaction domain for SOCS2 and also the SOCS box of SOCS2 as template for building the E3 ligase complex (31).

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, while 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 functionally modulated by Elongin B/C and that the availability of unbound Elongin B/C complex will determine CIS activity. So far, little is known on the mechanisms that determine free Elongin B/C levels in the cell and more detailed studies are clearly required. Elongin B/C can be part of different multiprotein complexes including the RNA polymerase II (Pol II) (62,63) machinery and a large subfamily of E3 ubiquitin ligases. This encompasses the VHL tumor suppressor complex (64) and over 70 proteins harboring a SOCS box in the human genome (according to Pfam database). As each of these could possibly sequester the Elongin B/C complex, it is clear that depending on induction pattern, subcellular localization and relative binding affinities of all these SOCS box containing proteins, a competition for Elongin B/C will occur. Of note, also viral genomes can encode proteins that recruit Elongin B/C, e.g. HIV-1 Viral infectivity factor (Vif) that suppresses the antiviral activity of APOBEC3G (65). We here also mention that inappropriately elevated SOCS (66-71) or Elongin C (72) 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, whilst no effect is seen for the interaction with MyD88. Perhaps, this observed Elongin B/C dependency may function as a ‘safety lock’ for ensuring complete suppression of signalling 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 like the 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 (36,73) may also suggest the high risk of unrestrained CIS activity.

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

1. Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D.,
Cheng, A., Himms-Hagen, J., Chan, C. C., Ramachandran, C., Gresser, M. J., Tremblay, M. L., and
Endocrinol 34, 339-351
5. ten Hoeve, J., de Jesus Ibarra-Sanchez, M., Fu, Y., Zhu, W., Tremblay, M., David, M., and Shuai,
1803-1805
Natl Acad Sci U S A 95, 10626-10631
10. Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita,
Biol Chem 276, 20703-20710
Neuroimmunol 109, 34-39
18. Narazaki, M., Fujimoto, M., Matsumoto, T., Morita, Y., Saito, H., Kajita, T., Yoshizaki, K., Naka,
19. Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A.,
Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., Taniguchi, T., Fujita, T.,
21. Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S.,
L., Cary, D., Richardson, R., Hausmann, G., Kile, B. J., Kent, S. B., Alexander, W. S., Metcalf, D.,
24. Kamura, T., Burian, D., Yan, Q., Schmidt, S. L., Lane, W. S., Querido, E., Branton, P. E.,

FOOTNOTES

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The abbreviations used are: JAK, Janus kinase; STAT, signal transducer and activator of transcription; SOCS, suppressor of cytokine signalling; CIS, cytokine inducible SH2 containing protein; SH2, src homology; MAPPIT, mammalian protein-protein interaction trap.
Table 1. Overview of the constructs used in the present study

Fig. 1. CIS inhibition of Epo and IL-3 signalling depends on Elongin B/C recruitment. A. Domain structure of CIS and amino-acid sequence alignment of the SOCS boxes of mouse SOCS1, SOCS2, SOCS3 and CIS. Conserved residues are highlighted by different colors in the sequence alignment. The B/C box and IPLN/LPXP motif, which respectively confer Elongin B/C and Cullin binding, are indicated. B. HEK293-T cells were transiently cotransfected with expression vectors for CIS, SOCS2 or their ΔB/C or ΔCul box deletion mutants, combined with the human EpoR and the STAT5-responsive β-casein-luciferase reporter. 24 h after transfection, cells were left untreated or were stimulated overnight with Epo (5 ng/ml) and luciferase measurements were performed in triplicate. Data are expressed as fold induction (stimulated/non stimulated ratio). Expression of the FLAG-tagged CIS and SOCS2 proteins was verified on lysates using anti-FLAG antibody. C. Ba/F3 cells were transiently electroporated with plasmids encoding CIS or CISΔB/C, combined with the STAT5-responsive pGL2-SPI2.1-luciferase reporter. 48 h after electroporation, the cells were starved and were left untreated or were stimulated overnight with mIL-3 (1ng/ml). Luciferase activities are represented as fold induction (stimulated/non stimulated ratio).

Fig. 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 left 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 main text for explanation of the MAPPIT technique. B. HEK-293T cells were transiently cotransfected with bait plasmids encoding the LR (YYF), the EpoR Y402 or the GHR Y595 motif and prey plasmids encoding RNF41 as positive control, CIS, SOCS or derived mutant constructs, combined with the STAT3 responsive pXP2d2-rPAPI-luciferase reporter. 24 h after transfection, cells were left untreated or were stimulated with leptin (100 ng/ml) or Epo (5ng/ml) overnight. Luciferase data of triplicate measurements are expressed as fold induction (stimulated/non stimulated ratio). The expression of the FLAG-tagged prey proteins were evaluated by immunoblotting using anti-FLAG antibody. C. (Phospho)peptide affinity chromatography. HEK-293T cells were transfected with FLAG-tagged CIS, SOCS2 or mutant derivatives. The lysates were incubated with phosphorylated or non-phosphorylated peptides corresponding to the Y402 motif of the EpoR. Specific protein binding was revealed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using the anti-FLAG antibody. D. HEK-293T cells were transiently cotransfected with bait plasmids encoding the GHR Y595 motif or MyD88 and prey plasmids encoding CIS or mutants, combined with the STAT3 responsive pXP2d2-rPAPI-luciferase reporter. MAPPIT signalling was assayed as described in Figure 2B. E. Verification of the interaction pattern of the CIS mutants. In the upper panel HEK293-T cells were transiently 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 pXP2d2-rPAPI-luciferase reporter. MAPPIT signalling was assayed as described in Figure 2B. In the bottom panels the bindings mode of the CIS mutants are demonstrated by co-immunoprecipitation experiments. Lysates of HEK-293T cells transfected with FLAG-tagged CIS, CISΔB/C or mutant derivatives were immunoprecipitated 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, CISΔB/C or CISΔCul and the lysates were immunoprecipitated with anti-E and then immunoblotted with anti-FLAG (right panel).

Fig. 3. Effect of Elongin B/C on CIS half-life. HEK293-T cells were transiently transfected with combinations of FLAG-tagged CIS, CISΔB/C or Elongin B/C. 24 h after transfection, cells were treated with cycloheximide (CHX) (20 µg/ml) for different time points. The lysates were revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody. CIS expression levels were detected and quantified using the Odyssey Infrared Imaging System (Li-Cor) and normalized for β-actin expression levels. The graph shows processed data of the degradation assay above in which the initial maximal expression was defined as 100%.
Fig. 4. Substrate interaction of other SOCS proteins is not Elongin B/C dependent. A. MAPPIT analysis of the binding modus of SOCS6 and SOCS7 (ΔB/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-rPAP1-luciferase reporter. MAPPIT signalling was assayed as described in Figure 2B. B. Co-immunoprecipitation analysis of the interaction between JAK2 and SOCS1(ΔB/C). HEK-293T cells were transiently transfected with combinations of JAK2 and E-tagged SOCS1, SOCS1ΔB/C or MyD88 as negative control. Cell lysates were immunoprecipitated with anti-E-tag and subsequently immunoblotted with anti-JAK2. C. Effect of the deletion of the B/C box on SOCS3 inhibition of EpoR signalling. HEK293-T cells were transfected with expression vectors for SOCS3, CIS or their ΔB/C deletion mutants, combined with the human EpoR and the β-casein-derived luciferase reporter gene. EpoR signalling was assayed as described in the legend of Figure 1B. D. (Phospho)peptide affinity chromatography to analyse the interaction between SOCS3 or its ΔB/C mutant and the Y985 motif of the LR. HEK-293T cells were transfected with FLAG-tagged SOCS3 or SOCS3ΔB/C and lysates were incubated with phosphorylated or non-phosphorylated peptides corresponding to the Y985 motif of the LR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody.

Fig. 5. CIS displays a lower affinity for Elongin C in comparison to SOCS2. A. Co-immunoprecipitation analysis of the interaction between CIS or SOCS2 and endogenous Elongin C. HEK-293T cells were transiently cotransfected with E-tagged CIS, SOCS2 or APOBEC3G as a negative control. Cell lysates were immunoprecipitated with anti-E-tag and subsequently immunoblotted with anti-Elongin C. B. MAPPIT competition assay in HEK293-T cells transiently expressing the pXP2d2-rPAP1-luciferase reporter and the Elongin C bait combined with the SOCS2 or CIS preys and increasing amounts of a competitor (CIS for the SOCS2 prey and SOCS2 for the CIS prey). MAPPIT signalling was assayed as described in Figure 2B.

Fig. 6. Elongin B/C levels can determine CIS activity. A. The bindings potency of the CIS prey depends on Elongin B/C levels in MAPPIT. HEK-293T cells were transiently cotransfected with expression vectors encoding the pXP2d2-rPAP1-luci reporter, the GHR Y595 bait and CIS or SOCS2 (ΔB/C) preys, with or without Elongin B/C co-expression. MAPPIT signalling was assayed as described in Figure 2B. B. Occurrence of 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.

Fig. 7. Homology models for CIS. Two different homology models were built for CIS, with a SOCS box orientation as in SOCS2 (homology model 1, panel A) or SOCS4 (homology model 2, panel B). In homology model 1 for CIS, Y253 forms a hydrogen bond with the buried C-terminal carboxyl group (panel C). The C-terminal residue L257 in CIS interacts with residues in the βG strand (P212) and the αB helix (V160, V164) of the CIS SH2 domain. In this model, mutating Y253 might lead to structure changes in the adjacent EF and BG loops. As these loops interact with the phosphopeptide substrate in the CIS homology model, this can affect substrate binding. In a model for CIS based on the SOCS4 structure (homology model 2), Y253 forms a hydrogen bond with D108 (panel D). R107 in CIS is the βB5 arginine, a residue that is critical in SH2 domain phosphopeptide binding (74). A CIS R107K mutant is dominant-negative for CIS, but still associates with IL-2Rα (75), reminiscent of the binding of our CIS mutants to MyD88. If the second model is correct, disruption of the hydrogen bond to D108 might affect the position of R107 and thus the affinity for phosphopeptide substrates.
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Figure 1

A  Pre-SH2 domain  SH2 domain  SOCS box

B

C

- 187 -
Figure 2

A

B

C

- 188 -
**D**

![Graph showing fold induction with data for different prey interactions.]

**E**

![Graph showing fold induction with data for different prey interactions.]

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Figure 3

The diagram shows the effects of CHX treatment on the levels of CIS, CISΔB/C, and CIS + Elongin B/C proteins over time. The graph depicts the percentage of CIS remaining at various time points (0, 1, 3, and 6 hours) for each condition. The CHX treatment is indicated for each time point, demonstrating the degradation of CIS over time compared to the control conditions. The β-actin band serves as a loading control.
Figure 4

A

B

C

D
Figure 5

A

E-tag: SOCS2  CIS  APOBEC3G
IP: anti-E
IB: anti-Elongin C

Total lysate: anti-E

B

Prey

Fold Induction

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<th>SOCS2 prey</th>
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<td>/</td>
<td>/</td>
<td>/</td>
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<td>+1µg CIS</td>
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<td>+0.3µg SOCS2</td>
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<tr>
<td>+0.3µg SOCS2</td>
<td>+0.3µg SOCS2</td>
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</table>

ElonginC bait
Figure 6

A

![Graph showing fold induction of different prey proteins.]

B

![Western blot images with labeled fractions and protein markers.]

- thyroglobulin (670kDa)
- γ-globulin (158kDa)
- ovalbumin (44kDa)
- myoglobin (17kDa)
Figure 7

A

B

C

D

SOCS box

EOCS box

helix B

helix B

I257

V164

V160

F104  P212  W82

Y253

C-terminal

R107

V83

V253

D108
CHAPTER 11: Versatility of the SOCS box domain

I. Review: The many faces of the SOCS box

The many faces of the SOCS box (Review)

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Short title: Versatility of the SOCS box

Key words: regulation, signal transduction, SOCS, SOCS box
ABSTRACT

The SOCS box is a structural domain found at the C-terminus of over 200 human proteins. It is usually coupled to a protein interaction domain such as an SH2 domain in case of the SOCS proteins. Most insights into the role of the SOCS box come from studies on these important regulators of cytokine signalling. A well established, general function of the SOCS box is the recruitment of Elongin B/C and Cullin proteins leading to the formation of an E3 ligase complex. SOCS proteins thus can negatively regulate signalling by marking tyrosine-phosphorylated cytokine receptors for proteasomal degradation. A similar mechanism was more recently uncovered for controlling SOCS activity itself, since SOCS2 was found to enhance the turnover of other SOCS proteins, thus restoring cytokine responses. The SOCS box can add unique features to individual SOCS proteins: it can function as an adaptor domain coupling activated receptors to downstream signalling pathways as was demonstrated for SOCS3, or as modulator of substrate binding by the SH2 domain in case of CIS. In this review we discuss these multiple roles of the SOCS box, which emerges as a versatile module controlling SOCS protein activity and cytokine signalling via multiple mechanisms.

INTRODUCTION

Cytokines are secreted proteins that regulate a broad range of biological processes as diverse as haematopoiesis, immune responses, cell development and growth. Binding of cytokines to cell surface receptors on target cells leads to multiple signalling events, including activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (figure 1) (reviewed in (Ihle et al., 1994; Leonard and O'Shea, 1998; Rawlings et al., 2004b)). Cytokine-induced signal transduction must be tightly regulated to avoid the detrimental consequences of excessive stimulation. Accordingly, several mechanisms exist to control the kinetics and magnitude of signalling at different levels. Three major classes of negative regulators are the protein tyrosine phosphatases (PTPs), protein inhibitors of activated STATs (PIAS) and suppressors of cytokine signalling (SOCS) proteins (reviewed in (Greenhalgh and Hilton, 2001)).
Cytokine binding to its receptor complex induces reorganisation of the receptor chains, which leads to juxtaposition and activation of the JAKs by cross-phosphorylation. JAKs phosphorylate the cytoplasmatic domains of receptors, creating docking sites for cytoplasmatic proteins such as STATs. Following phosphorylation by the action of the JAKs, STATs form dimers and migrate to the nucleus to induce target genes, including those that express the SOCS. Acting in a negative feedback loop, SOCS will then attenuate receptor signalling by different mechanisms (see text).
The SOCS family consists of eight proteins, SOCS1-7 and CIS, of which the expression is induced in response to a wide range of cytokines, growth factors and hormones (Adams et al., 1998; Dogusan et al., 2000; Krebs and Hilton, 2003; Yasukawa et al., 2000). They were identified as inhibitors of the JAK-STAT signalling pathway, operating as part of a classical negative feedback loop (Fujimoto and Naka, 2003; Greenhalgh and Hilton, 2001; Krebs and Hilton, 2001). SOCS proteins display a similar domain architecture with an N-terminal region of variable length, a central Src homology 2 (SH2) domain which is involved in binding of phosphotyrosine motifs and a conserved C-terminal domain, known as the SOCS box (figure 2).

**Figure 2: Domain structure of the SOCS protein family**
The major structural characteristics of the SOCS family are the presence of two domains with relatively well conserved AA sequence: a SH2 domain in the middle portion and a SOCS box at the C-terminus. Only SOCS1 and -3 possess a KIR immediately upstream of the SH2 domain. Conserved tyrosines in the SOCS box are indicated by a black line.

SOCS proteins modulate cytokine receptor responses by different mechanisms (figure 1). First, they can suppress signalling by competing with other signal transducers for binding to phosphorylated motifs of the activated receptor via their SH2 domain (Ram and Waxman, 1999; Yoshimura et al., 1995). Second, a small kinase inhibitory region (KIR) found in the N-terminal domain of SOCS1 and SOCS3 inhibits the activity of JAKs by acting as a pseudo-substrate (Yasukawa et al., 1999). SOCS1 binds directly to the phosphorylated activation loop of JAK2,
whereas SOCS3 shows only weak affinity for JAK2 and is thought to bind to the receptor in close proximity of the kinase (Giordanetto and Kroemer, 2003; Nicholson et al., 2000; Sasaki et al., 2000). Third, SOCS proteins can regulate signal transduction by linking their substrates to ubiquitination and proteasomal degradation via the SOCS box. This latter domain was found to recruit Elongin B and C (B/C) proteins (Kamura et al., 1998; Zhang et al., 1999) which can associate to a Cullin-Rbx module reconstituting an E3 ubiquitin ligase complex (Kamura et al., 2001). The involvement of the SOCS box in proteasomal degradation will be discussed in more detail in the next section. In addition to proteasomal turnover, SOCS proteins can also direct the internalisation and routing of cytokine receptors as was for example demonstrated for the growth hormone (GH) and granulocyte-colony stimulating factor (G-CSF) receptors (Irandoust et al., 2007; Landsman and Waxman, 2005). SOCS proteins not only control kinetics and magnitude of signalling but can also be involved in the shaping of cytokine responses. For example, SOCS3 that is induced by different cytokine receptors regulates both the quantity and type of STAT signalling generated from the IL-6R. In the absence of SOCS3, IL-6 induces a wider transcriptional response that is dominated by interferon (IFN)-like gene expression owing to an excess of STAT1 phosphorylation (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003). SOCS proteins thus regulate cytokine receptor signalling at different levels and by multiple complementary mechanisms, the importance of which differs according to both the individual SOCS molecule and the triggered cytokine pathway.

**THE ROLE OF THE SOCS BOX IN TARGET PROTEIN DEGRADATION**

Protein degradation by the ubiquitin-proteasome pathway requires a cascade of enzymatic reactions involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and finally an E3 ligating enzyme. This latter enzyme defines substrate specificity and covalently associates ubiquitin to lysine side chains in the substrate. The Elongin C-Cullin-SOCS box (ECS)-type E3 ubiquitin ligases include among others the Von Hippel-Lindau (VHL) tumor suppressor protein and the SOCS family members, that fulfill the role of substrate recognition unit of the complex (Ivan and Kaelin, 2001; Kibel et al., 1995; Kile et al., 2002). The SOCS box domain of
these proteins mediates the interaction with Elongin C by the B/C box, a xxLxxxCxxx (A/I/L/V) conserved sequence (Aso et al., 1996; Duan et al., 1995; Kamura et al., 1998; Kibel et al., 1995). Elongin B binds Elongin C and this dimer acts as a linker that bridges the substrate recognized by the SOCS box protein to a Cullin scaffold protein. This association with a Cullin protein is further supported by a conserved Cullin box motif, located downstream of the B/C box in the SOCS box (Kamura et al., 2004). Cullin in turns recruits a RING finger-containing protein Rbx, thereby completing the assembly of the E3 ligase complex (figure 3A) (Iwai et al., 1999; Pause et al., 1997).

Figure 3: Involvement of the SOCS box in protein degradation
(A.) Mechanism by which SOCS can target associated proteins for proteasome-mediated destruction. (B.) Proposed mechanism whereby SOCS themselves can be targeted for degradation by E3 recruitment or other SOCS proteins.

SOCS can act as the substrate recognition component of an ECS-type E3 ubiquitin ligase to regulate the half-life of proteins. First of all, signal transduction of several cytokines is prolonged in the presence of proteasome inhibitors (Callus and Mathey-Prevot, 1998; Verdier et al., 1998). More specifically, it was demonstrated that SOCS1 promotes the ubiquitination and turnover of JAK2 and the TEL-JAK2 oncogene in a SOCS box dependent fashion (Frantsve et al., 2001; Kamizono et al.,...
Chapter 11: Versatility of the SOCS box domain

2001; Ungureanu et al., 2002) while SOCS3 can target receptors for proteasomal degradation as it accelerates destruction of CD33 and sialic acid-binding immunoglobulin-like lectin (Siglec) 7, members of the Siglec family of receptors (Orr et al., 2007a; Orr et al., 2007b). This proteolytic activity is not restricted to the receptor complex or associated JAKs but also targets substrates as diverse as the signal transducer VAV, the p65/RelA subunit of NF-κB, the Toll-like receptor (TLR) adaptor Mal and the E7 protein of Human papilloma viruses (HPV) in case of SOCS1 (De Sepulveda et al., 2000; Kamio et al., 2004; Mansell et al., 2006; Ryo et al., 2003). Furthermore, SOCS1 and SOCS3 can both promote destruction of Insulin Receptor Substrate (IRS) 1 or IRS2 and focal adhesion kinase (FAK) (Liu et al., 2003; Rui et al., 2002). Compellingly, an essential role for the SOCS box of these SOCS members was demonstrated in vivo. Transgenic mice expressing a SOCS box deletion variant of SOCS1 or SOCS3 exhibit impaired regulation of respectively INFγ (Zhang et al., 2001) and Granulocyte-colony stimulating factor (G-CSF) signalling and response to inflammatory stimuli (Boyle et al., 2007).

In contrast to SOCS1 and SOCS3, CIS and SOCS2 cannot directly inhibit JAK activity thus implicating a stronger dependency on substrate competition or proteasomal degradation of target proteins. CIS association was demonstrated to induce proteasome-dependent degradation of the erythropoietine receptor (EpoR) and growth hormone receptor (GHR) (Landsman and Waxman, 2005; Ram and Waxman, 2000; Verdier et al., 1998). The SOCS box of SOCS2 appeared to be crucial for the negative regulation of GH signalling (Greenhalgh et al., 2005). The crystal structure of the SOCS2-Elongin B/C complex revealed a SOCS box ubiquitin ligase architecture similar to the one of VHL (Bullock et al., 2006), further supporting a role for SOCS2 in E3 ligase activity. The SOCS box shows an extensive interaction with Elongin C, centered around the B/C box motif. The SOCS box also directly interacts with Elongin B (figure 4A and 4B).

The remaining SOCS members SOCS4-SOCS7 have been less extensively examined. SOCS4 and SOCS5 are induced upon epidermal growth factor (EGF) stimulation and subsequent SOCS box dependent turnover of EGFR was reported, thereby inhibiting the mitogenic signalling (Kario et al., 2005; Nicholson et al., 2005). Also, the structural resolution of the SOCS4-Elongin B/C complex revealed a
molecular basis for SOCS-mediated EGFR degradation (Bullock et al., 2007). SOCS6 was found to interact with haem-oxidized IRP2 ubiquitin ligase 1 (HOIL1), thereby driving ubiquitination and degradation of associated proteins (Bayle et al., 2006). This observation illustrates that SOCS proteins may interact with different E3 ubiquitin ligases in addition to a common ECS-based complex. Even though more direct evidence for SOCS ubiquitin ligase activity remains to be demonstrated for the latter SOCS members, together these data point to the key role of the SOCS box in mediating protein turnover as a general SOCS mechanism of action. While many receptor complexes recruit SOCS, only a few of these receptors or their associated JAK/STAT proteins are known targets for SOCS mediated degradation. This may indicate a broader role for SOCS proteins in the regulation of downstream substrates. Accordingly, proteasomal targeting of substrates as diverse as other SOCS proteins, TLR adaptors and viral proteins have been reported (Kamio et al., 2004; Mansell et al., 2006; Piessevaux et al., 2006; Tannahill et al., 2005).

The crystal structures of SOCS2 and SOCS4 with Elongins B and C show a remarkable difference. While the interactions of the Elongins with the SOCS box are very similar, the SOCS box itself assumes a different orientation versus the SH2 domain (figure 4A and 4B). Based on the structural information of SOCS2, it was proposed that in SOCS1-3 and CIS the C-terminus is buried in the core of the structure where it stabilizes the interaction between the SH2 domain and the SOCS box. This packing partially exposes the N-terminal ESS providing better accessibility for the SOCS1 and SOCS3 KIR domain. This domain organization precludes C-terminal extensions and explains the strictly conserved length of the C-terminal parts in CIS and SOCS1-3 (Bullock et al., 2006). In contrast, the SOCS4-7 subclass contains extended C-termini and there the N-terminal ESS helix is buried in the SOCS box / SH2 interface to fulfill an equivalent packing role as could be demonstrated in the SOCS4 structure. The function of the C-terminus is then redefined to stabilize an interface with the N-terminal domain (Bullock et al., 2007).
Figure 4: Structure of SOCS and SOCS-Elongin B/C complexes
A. Structure of the SOCS2-Elongin B-Elongin C complex.  B. Structure of the SOCS4-Elongin B-Elongin C complex. In A & B, the SOCS SH2 and pre-SH2 domain is colored orange, the SOCS box is colored red, Elongin B is yellow, Elongin C is green. In the SOCS4 complex, the SH2 domain differs from its orientation in the SOCS2 complex.
C & D. Accessible surface plot of SOCS4, when the SOCS4 molecule is oriented as in the ribbon plot below (E)(In E, the SOCS box is colored red). In C, the surface is colored according to the atom colors. In D, the surface is colored according to residue conservation in the human SOCS proteins. Strictly conserved residues are colored red, unconserved residues are colored dark blue. The Elongin binding site (circled area) is hydrophobic and highly conserved.
THE SOCS BOX AND SOCS PROTEIN TURNOVER

Effect of E3 ligase environment on SOCS stability

Next to its role in the degradation of associated substrates, the SOCS box may also be involved in the regulation of the expression levels of the SOCS proteins themselves. Reports in the literature are however somewhat paradoxical indicating that the functional significance of the interaction between Elongin B/C and the SOCS box is complex. Some data suggest that the Elongin B/C complex constitutively links SOCS to E3 ligase activity, thereby promoting its degradation by the proteasome. Accordingly, mutations or post-translational modifications of SOCS proteins that disrupt Elongin B/C recruitment have a positive effect on protein stability as has been shown for SOCS1 (Chen et al., 2002; Kamura et al., 1998)\[Lirnander, 2004 #156\], SOCS3 (Sasaki et al., 2003; Zhang et al., 1999) and CIS (Ram and Waxman, 2000; Verdier et al., 1998). Overall, it appears that the proteasomal pathway plays a key role in SOCS protein down-modulation (Bayle et al., 2006; Narazaki et al., 1998; Sasaki et al., 2003; Verdier et al., 1998; Zhang et al., 1999). In addition, CIS and SOCS3 are found to be degraded concomitantly with their GHR or Siglec receptor target respectively (see above), suggesting a stimulation-dependent mechanism for SOCS turnover (Orr et al., 2007a; Orr et al., 2007b; Ram and Waxman, 2000). In contrast, the interaction between the SOCS box and Elongin B/C has also been shown to stabilize SOCS proteins (Haan et al., 2003; Hanada et al., 2001; Kamura et al., 1998; Narazaki et al., 1998; Zhang et al., 1999; Zhang et al., 2001). In the same line, the VHL protein required Elongin B/C binding for correct folding and stable expression (Feldman et al., 1999; Kamura et al., 2002; Melville et al., 2003). The interaction of the SOCS box with both Elongins is dominated by hydrophobic interactions. Removing the Elongins in the crystal structure of SOCS2 or SOCS4 exposes a large hydrophobic surface patch in both Elongin C and in the SOCS box (figure 4C). It is therefore not unlikely that the structure of the SOCS box will be unstable and undergo structural changes in the absence of Elongins. No structure of a SOCS box in the absence of Elongins has been determined: the crystal structures of SOCS2, SOCS4 and VHL were only determined upon co-crystallization with Elongin B and C (Bullock et al., 2006; Bullock et al., 2007; Stebbins et al., 1999). Together, these observations imply that
assembly of the E3 ligase induces structural reorganization and stabilization of the
different components.

While the cytokine-dependent regulation of SOCS synthesis is well
documented, the mechanisms controlling SOCS turnover remain elusive with a
paradoxical role for the SOCS box. Conceivably, stabilization by Elongin B/C
recruitment represents a mechanism whereby SOCS proteins are themselves
prevented from proteasomal degradation. This protection may allow SOCS proteins
molecules to function properly in an ubiquitin ligase complex. At some point
however, negative control is required for SOCS turnover which could occur via
either co-degradation with the targeted protein or by the degrading action of another
SOCS member.

**SOCS cross-modulation**

Emerging evidence points to regulatory cross-talk between SOCS family
members. SOCS protein down-modulation is likely necessary to restore cellular
sensitivity. Initially, it was found that SOCS2 exerts a dual effect on GH signal
transduction: low SOCS2 levels moderately suppressed GH actions whereas higher
levels restored and even enhanced signalling by blocking the inhibitory effects of
other SOCS proteins (Favre et al., 1999; Greenhalgh et al., 2002). This dual role of
SOCS2 was further supported by the observation that SOCS2-deficient mice
exhibited an overgrowth phenotype (Metcalf et al., 2000), while SOCS2 transgenic
mice also grew significantly larger than their wild type littermates (Greenhalgh et al.,
2002). Interference of SOCS2 with other SOCS proteins has been observed in
several cytokine receptor systems including prolactin receptor (PRL) (Dif et al.,
2001; Pezet et al., 1999), IL-2 and IL-3 (Tannahill et al., 2005), IFN type I and leptin
signalling (Lavens et al., 2006; Piessevaux et al., 2006). A stimulatory role for
SOCS2 was also proposed in mesenchymal precursor cells where it could
potentiate osteoblast differentiation through upregulation of JunB expression,
possibly through its negative effect on other SOCS proteins (Ouyang et al., 2006). In
vivo observations further support a stimulatory role of SOCS2. Proteasomal
degradation of SOCS1 induced by endogenous SOCS2 was proposed as the
molecular mechanism behind the constitutively activated phenotype associated with VHL-mediated Renal Cell Carcinoma (Wu et al., 2007). SOCS2 upregulation was found to correlate with advanced stages of chronic myeloid leukemia (CML) (Schultheis et al., 2002; Zheng et al., 2006) or acute myeloid leukemia (AML) (Faderl et al., 2003)(personal communication, Dr. I Touw), possibly by inhibiting the tumor suppressor function of other SOCS proteins.

Although the exact nature of these SOCS interactions and consequent cross-regulation is yet to be determined, the effect relied on the presence of the SOCS box of the targeted SOCS and the B/C box of SOCS2 (Piessevaux et al., 2006). Using MAPPIT we found that SOCS2 (and SOCS6 and -7) can bind with all members of the SOCS protein family and the SOCS box of the targeted SOCS appears to be implicated. This might indicate that SOCS2 can promote turnover of associated SOCS molecules by linking them to proteasomal activity (figure 3B). Mapping SOCS residue conservation on the surface of the SOCS structures shows that the Elongin binding site is the only conserved feature in the surface of SOCS proteins, indicating the importance of Elongin binding and a similar way of Elongin binding for all SOCS proteins (figure 4D). The conserved patch in the SOCS box is a possible SOCS2 binding site. SOCS2 may also compete for recruitment of the Elongin B/C proteins to other SOCS proteins, resulting in destabilization of these other SOCS proteins lacking the complex. In analogy with SOCS2, SOCS6 may also potentiate cytokine signalling by controlling SOCS protein stability (Piessevaux et al., 2006). This latter finding might explain the improvement in glucose metabolism observed in SOCS6 transgenic mice (Li et al., 2004). Furthermore, the *Drosophila melanogaster* SOCS protein SOCS44A, which is most similar to SOCS6, was found to enhance the activity of the EGFR/MAPK signalling cascade, providing further evidence for a positive role for SOCS6 in cytokine signalling (Rawlings et al., 2004a).

In line with such a SOCS cross-regulatory mechanism is the sequential induction pattern of different SOCS molecules. In contrast to CIS, SOCS1 and SOCS3, which are typically induced in a rapid and transient manner upon receptor activation, expression of SOCS2 usually occurs later after cytokine stimulation and is more prolonged (Adams et al., 1998; Brender et al., 2001; Pezet et al., 1999; Tannahill et al., 2005). Accordingly, the cytokine induced levels of some SOCS
members might act to restore cellular sensitivity for subsequent stimulation by suppressing the inhibitory effects of other SOCS proteins.

*Other mechanisms regulating SOCS turnover*

Next to the effects of Elongin B/C and the modulation by other SOCS proteins, the half-life time of SOCS molecules can be controlled by other protein-protein interactions. SOCS1 levels are negatively regulated by association with the tripartite motif (TRIM)8/Glioblastoma Expressed RING-finger Protein (GERP) which is a putative E3 ubiquitin ligase (Toniato et al., 2002), while SOCS6 is stabilized by the Ring-finger HOIL-1 protein (Bayle et al., 2006). Phosphorylation is also implicated in the control of SOCS turnover. SOCS1 expression levels are positively regulated through phosphorylation by the Pim serine/threonine kinase (Chen et al., 2002). In contrast, JAK-dependent phosphorylation of SOCS3 at two tyrosine residues in the SOCS box correlates with disrupted Elongin C interaction and accelerated SOCS3 degradation (Haan et al., 2003). It remains to be determined whether other SOCS can also be modulated by phosphorylation. Given the high degree of conservation of SOCS box tyrosine residues this mechanism may also occur for other SOCS proteins, but evidence for this is lacking so far. An unstructured PEST (proline-, glutamic acid-, serine and threonine rich) motif in the SH2 domain of SOCS3 can negatively regulate its protein stability (Babon et al., 2006). This multitude of mechanisms controlling SOCS stability suggests that the turnover of SOCS must be crucial in cytokine mediated responses.

**ADAPTOR FUNCTIONS OF THE SOCS BOX**

The SOCS box functions primarily as a linker that associates substrate binding domains such as the SH2 domain for SOCS proteins with the ubiquitin ligase components Elongin C and B (Hilton et al., 1998). In addition, this domain also couples SOCS actions to other downstream signalling pathways like the MAPK pathway. SOCS3 is phosphorylated on C-terminal tyrosines in response to insulin, IL-6 and many growth factors including IL-2, Epo, EGF and PDGF (Cacalano et al.,
Upon stimulation by the latter growth factors phosphorylated SOCS3 can interact with the Ras inhibitor p120 RasGAP, thereby inhibiting its functions. This way, SOCS3 maintains ERK activation and ensures cell survival and proliferation through the Ras/MAPK pathway (Cacalano et al., 2001). As phosphorylated SOCS3 was still able to block STAT5 activation, SOCS3 can act as a molecular switch turning off JAK-STAT mediated signals while sustaining ERK activation through the same receptor. Tyrosine phosphorylation of the SOCS box of SOCS3 was also found to mediate association and activation of the adaptor proteins Nck and Crk-L, which are known to couple activated receptors to multiple downstream signalling pathways and the actin cytoskeleton (Sitko et al., 2004). The effects of SOCS3 phosphorylation induced by insulin and IL-6 remains unclear but it can be envisioned that also here binding sites for SH2-containing molecules are created, thereby providing a link to other signalling systems (Peraldi et al., 2001; Sommer et al., 2005).

Similar to SOCS3, other SOCS members posses a conserved tyrosine residue in the SOCS box (Hilton et al., 1998) and many of these are in a potential SH2 domain-binding motif. It remains to be elucidated how these tyrosines contribute to SOCS function. In this context, CIS was reported to promote T cell receptor mediated proliferation and to prolong survival of activated T cells. The latter responses are dependent on increased MAPK activation and a direct interaction of CIS and protein kinase Cθ (Li et al., 2000). It remains to be determined if the SOCS box is implicated in these regulatory events.

THE SOCS BOX CAN PARTICIPATE IN SUBSTRATE RECOGNITION

A SOCS box can be a critical determinant for substrate recognition, as we recently demonstrated for CIS. The Elongin B/C recruitment site (B/C box), as well as a conserved C-terminal tyrosine (Y253) are required for CIS binding to EpoR, GHR and leptin receptor motifs (Lavens et al., 2007; Uyttendaele et al., 2007)(Piessevaux et al., submitted to JBC). Indirect effects on the structural integrity of CIS due to deletion of these sites can be excluded, as SOCS box independent interactions were observed with the unrelated TLR adaptor MyD88 (Lavens et al., 2007)(Piessevaux et al., submitted to JBC). This SOCS box dependency appeared
Chapter 11: Versatility of the SOCS box domain

to be unique for CIS, as none of the other examined SOCS members required Elongin B/C association or the homologous tyrosine for receptor substrate interaction. At present 70 proteins harboring a SOCS box have been described in the human genome (according to Pfam), possibly their substrate binding could also be controlled by Elongin B/C recruitment.

We could show that under physiological conditions CIS can exist in an uncoupled form, lending further support to a regulatory mechanism by which free CIS can be bound and regulated by the cellular Elongin B/C levels (Piessevaux et al., submitted to JBC). This way, the SOCS box may represent a regulatory on/off switch acting on the SH2 domain of CIS thereby controlling receptor binding. Accordingly, a CIS variant with a defective SH2 domain (CIS R107K) behaved as a dominant negative variant (Aman et al., 1999; Ram and Waxman, 2000) and we propose that this effect is due to the scavenging of Elongin B/C by the mutant resulting in loss of substrate binding by endogenous CIS. Our observation that Elongin B/C association cannot be uncoupled from CIS receptor binding further supports a major contribution for the recruitment of E3 ligase activity in CIS-mediated inhibition. This Elongin B/C-dependency also implicates that CIS activity may be suppressed by SOCS2 in a dual way. SOCS2 that is expressed at a later stage may scavenge Elongin B/C from CIS leading to loss of receptor interaction. Subsequently, SOCS2 can bind with uncomplexed CIS resulting in proteasomal targeting of CIS. Further studies are needed to establish the physiological significance of this modulatory mechanism. Given the important physiological processes in which CIS takes part such as the GHR-mediated growth or EpoR-dependent haematopoiesis, this molecular on/off switch may constitute a safety lock to ensure complete inhibition of cytokine activity since only CIS molecules that are able to recruit the E3 ligase activity will associate with the receptor.

MEDICAL IMPLICATIONS

Aberrant control of SOCS protein function, e.g. by deregulated expression levels, can contribute to several pathologies. Certain pathogens have developed strategies to induce the host SOCS system for manipulating cytokine signalling and
evading immune counter actions (Alexander et al., 1999; Moutsopoulos et al., 2006; Stoiber et al., 2001; Zimmermann et al., 2006). Alterations in SOCS protein levels, and more specifically SOCS3, has been implicated in the pathogenesis of various inflammatory diseases including rheumatoid arthritis (RA), Crohn’s disease and inflammatory bowel diseases (IBD) (Egan et al., 2003; Isomaki et al., 2007; Lovato et al., 2003; Rakoff-Nahoum et al., 2006; Suzuki et al., 2001). A prominent role for increased SOCS3 levels in leptin resistance and consequent obesity was also reported (Bjorbaek et al., 1998; Dunn et al., 2005; Howard et al., 2004; Mori et al., 2004). Furthermore, SOCS1 and SOCS3 might be players in the development of insulin resistance associated with type 2 diabetes (Emanuelli et al., 2001; Emanuelli et al., 2000; Kawazoe et al., 2001; Ueki et al., 2005; Ueki et al., 2004). Upregulated SOCS1 and SOCS3 expression also seems to be responsible for the unresponsiveness to IFN therapy in the treatment of hepatitis C virus infection or leukaemia (Bode et al., 2003; Roman-Gomez et al., 2004; Sakai et al., 2002). Compellingly, development and progression of tumors in various human cancers was correlated with both inactivation (Farabegoli et al., 2005; Galm et al., 2003; He et al., 2003; Komazaki et al., 2004; Nagai et al., 2003; Watanabe et al., 2004; Wikman et al., 2002; Yoshikawa et al., 2001) and inappropriate upregulation of certain SOCS proteins (Evans et al., 2007; Haffner et al., 2007; Komyod et al., 2007; Raccurt et al., 2003; Roman-Gomez et al., 2004). As mentioned above, increased expression of SOCS2 in malignancies like chronic myeloid leukemia (CML) (Schultheis et al., 2002; Zheng et al., 2006), could contribute to transformation by negative interference with other SOCS molecules that normally would suppress tumor development.

Manipulation of SOCS protein function might provide novel therapeutic options in the treatment of these disorders. Besides administration of SOCS proteins or mimetics and the specific downregulation of SOCS expression by siRNA or antisense approaches, the emerging knowledge about SOCS regulation may provide a structural basis for SOCS inhibitor design. The SOCS protein structure reveals specific domains that can be targeted to alter their function and expression: these include the SH2 domain to modulate association with other signalling molecules and the SOCS box to control SOCS stability and actions. Definitely, further insights in the molecular mechanisms behind SOCS regulation will be
important to completely understand their control of signalling pathways and their relationship with pathologies.

CONCLUDING REMARKS

Through their regulation of diverse signal transduction pathways, SOCS proteins are involved in a variety of crucial processes including immune functions, haematopoeiesis, growth and metabolism. Accordingly, regulation of SOCS functions itself is subjected to tight control. Here a complex role for the SOCS box emerges (figure 5).

Figure 5: Versatility of SOCS functions
The different domains of the SOCS box mediate distinct interactions and functions. Some of them are specific for some SOCS members (e.g. KIR-dependent inhibition of JAK activity by SOCS1 and SOCS3) while others appear to be general (competition for receptor motifs). The SOCS box is involved in functions as diverse as target degradation, control of SOCS stability and receptor interaction.

First, the domain is involved in the destruction of targets by linking them to the proteasomal machinery. Second, it may also protect SOCS from this proteasome mediated turnover. Third, the SOCS box is involved in a cross-
regulatory mechanism in which a restricted set of SOCS proteins can interfere with the inhibitory actions of other members of the family. As the latter mechanism will exert a negative effect on SOCS half-life time, this also may provide an explanation for the apparent duality of the SOCS box on SOCS stability. Together, these data suggest that the association between the SOCS box and the Elongin B/C complex could function, at least in part, to fine-tune the concentration and actions of SOCS proteins in cells. Finally, the SOCS box can also be involved in a unique regulatory mechanism controlling SH2 domain functions as was demonstrated for CIS.

Since SOCS proteins are involved in several important human pathologies, a full understanding of the mechanisms controlling their activity is of great importance. This should not be underestimated given the remarkably different contribution of the SOCS box in the activity pattern of individual SOCS proteins. Full insight into their finely tuned activity will require detailed analysis of the mechanisms controlling their expression levels and interaction patterns.
Chapter 11: Versatility of the SOCS box domain
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Chapter 11: Versatility of the SOCS box domain


Chapter 11: Versatility of the SOCS box domain


Conclusions and future prospects

Interactions between proteins are fundamental to virtually every biological process. Therefore, the characterisation of such protein-protein interactions is imperative in the understanding of cellular mechanisms and makes them new targets for drug design. Proteins can function as components of large, highly structured complexes such as ribosomes, enhanceosomes or the proteasome complex. But protein associations also contribute to the regulation of signal transduction, which are generally of a temporary nature. Full characterisation of a particular protein-protein interaction or the comprehensive large-scale determination of the proteome within a particular organism necessitates the complementary use of different methodologies. Each approach will add unique advantages and opportunities but will also have specific drawbacks. As discussed in chapter 4, a broad range of both biochemical and genetic techniques have been developed to study protein-protein interactions. Biochemical methods have the advantage that signalling complexes can be analysed in its entirety. However, the inevitable lysis step in these approaches disrupts the normal cellular context, which can lead to false positive interactions between proteins normally residing in separate cellular compartments. Also, protein interactions involved in dynamic intracellular processes including signal transduction may be too weak or transient to be detected with biochemical techniques. Genetic approaches use hybrid bait and prey molecules, of which interaction generates a measurable signal. The most commonly used, yeast-two-hybrid, is cost-effective, easy to implement and scalable. This method suffers from two major intrinsic limitations: interactions between mammalian proteins often require post-translational modifications which are hard to reproduce in yeast cells and as the bait-prey interaction need to occur in the nucleus this will exclude the analysis of membrane bound proteins. In order to work in an optimal physiological context, a variety of two-hybrid systems in mammalian cells, have been developed. MAPPIT is such a technique that relies on signal transduction via type I cytokine receptors ([www.mappit.be](http://www.mappit.be)) (Eyckerman et al., 2001). Interaction of bait and prey hybrids leads to functional complementation of a signalling-deficient receptor by STAT3.
recruitment sites in the prey protein. Throughout this thesis MAPPIT was extensively used to study (cross)regulation by SOCS molecules. The technology was also expanded by application of different variants and by working in diverse cellular contexts, demonstrating the versatility of MAPPIT as an analytical tool.

Since cytokine signalling cascades are vital to many physiological processes, strict control of these pathways is equally important. SOCS proteins are an important family of regulators of these cytokine responses. Biochemical and genetic studies have provided profound insights into the modes of action and selectivity by which the different SOCS proteins regulate distinct signal transduction pathways. Anyhow, the mechanisms underlying SOCS functions need to be further elucidated. Better insight into these may help to understand the regulatory mechanisms underlying differential cellular responsiveness to cytokines. The importance of this is underscored by the contribution of inappropriately regulated SOCS levels to several pathologies.

In the first part of this thesis we focussed on the role of SOCS proteins in modulation of leptin signalling. This cytokine-like hormone is crucially involved in body weight regulation by communicating the status of body fat stores to the hypothalamus. Aberrantly high leptin levels are associated with the majority of the obesity cases, pointing to a central failure to respond correctly to the leptin signal. Accumulating evidence indicates that alterations in cellular LR signalling, with a key role for SOCS3, have a major contribution in this leptin resistance (Munzberg et al., 2005). We found that CIS and SOCS2 are new interaction partners of the LR. In general, the closely related CIS and SOCS2 proteins display a great overlap in their binding mode with cytokine receptors. By using MAPPIT and peptide affinity chromatography we observed a differential interaction pattern of CIS and SOCS2 with phosphotyrosine motifs of the LR. Whereas both CIS and SOCS2 associate with the P-Y1077 position, only CIS interacts with the more membrane proximal P-Y985. We analysed the functionality of SOCS2 in the context of LR signalling and showed that it can impede STAT5 recruitment. We further observed that SOCS2 interferes with CIS association at P-Y1077 and unexpectedly also at the P-Y985 position, although SOCS2 itself does not interact with this tyrosine. Since SOCS2
was found to associate with CIS and the interfering effect appeared to be dependent on Elongin B/C recruitment to SOCS2, proteasomal degradation of CIS is likely involved. This observed cross-regulation between SOCS proteins was analysed in more detail in the second part of this thesis (see further). We conclude that besides the well characterized inhibitory actions of SOCS3, other SOCS proteins interact with the LR and may be implicated in the modulation of its signalling. Upregulation of CIS or SOCS2 expression is not detectable in the hypothalamus upon leptin administration in mice (Bjorbaek et al., 1998), but it should be considered that SOCS proteins can mediate cross-talk between different signalling pathways. Also their expression may be relevant in peripheral cell types and accordingly, SOCS2 and CIS expression upon leptin stimulation were found in RINm5F insulinoma cells (Lavens et al., 2006).

Leptin has been increasingly recognized as a regulator of peripheral functions like immune responses, reproduction, angiogenesis and haematopoiesis. Nevertheless, signalling events induced by leptin in haematopoietic progenitor cells remain far from clear. In order to gain more insight in this issue, we studied the interaction partners of the LR in a haematopoietic background. We adapted the MAPPIT strategy to this cellular context that is characterized by prevalent STAT5 signalling by exchanging the gp130 part of the preys by a portion of the cytoplasmatic tail of the βc-receptor containing STAT5 interaction sites. This so-called βc-MAPPIT was successfully used in different haematopoietic cell lines. The interaction of different signalling molecules with tyrosine motifs of the LR was tested. Known interactions could be confirmed: we found binding of STAT5 to the P-Y1077 and of SOCS3 to the P-Y985 and P-Y1077 motif (Eyckerman et al., 2000; Gong et al., 2007; Hekerman et al., 2005). Furthermore, we reported the involvement of the phospholipase C pathway in haematopoietic leptin signalling by showing the interaction of PLCγ with the P-Y1077. Since our previous interaction studies with the classical MAPPIT method demonstrated binding of CIS and SOCS2 to the LR in HEK-293T cells, we next examined all SOCS family members on LR interaction. CIS and SOCS7 were found to both interact with the P-Y985 and P-Y1077 motifs, while SOCS2 and -6 only bind to the P-Y1077 of the LR. Association of SOCS2, -6 and -7 at P-Y1077 may be indirect as it was recently reported that IRS4 functions as an adaptor of the LR at this position, recruiting different proteins including these

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SOCS members (Wauman et al., 2007). The inability to distinguish direct from indirect interactions is inherent to all two-hybrid methods and full proof of this requires in vitro interaction analysis of the purified recombinant binding partners. These data further support the implication of other SOCS molecules, besides SOCS3, in the modulation of leptin signalling. Also, our study provides a basis for more detailed functional analysis of leptin signalling in haematopoietic cells.

The second part of this thesis deals with the versatile functions of the SOCS box on the regulation of SOCS proteins and cytokine signalling. A growing body of evidence indicates that the functions of some SOCS proteins may not be simply inhibitory. In this respect, SOCS2 was described as a dual regulator, exerting both inhibitory and stimulatory effects on GH signalling in vitro and in vivo (Favre et al., 1999; Greenhalgh et al., 2002; Metcalf et al., 2000). We unravelled the underlying mechanism of this paradoxal effect. First we showed that SOCS2 antagonizes the inhibition of SOCS1 and SOCS3 in a SOCS box-dependent manner, thereby restoring signalling via the GHR, LR and IFN type I receptor. This interfering capacity of SOCS2 was found to depend on Elongin B/C recruitment. Accordingly, degradation of SOCS1 was promoted by SOCS2 but not by the Elongin B/C recruitment-deficient mutant, suggesting that SOCS2 targets other SOCS proteins for proteasomal degradation. Using MAPPIT we demonstrated that SOCS2 can bind to all members of the SOCS protein family. We finally provide evidence for the involvement of SOCS6 in a similar interfering mechanism. This might reflect a crucial physiological role of SOCS2 in restoring cellular responsiveness after cytokine activation. It can be presumed that SOCS2 requires a threshold concentration to act on SOCS protein degradation. In line with this, SOCS2 expression usually occurs at later time points compared with CIS, SOCS1 and -3 and is more prolonged (Adams et al., 1998; Brender et al., 2001; Pezet et al., 1999; Tannahill et al., 2005). Elevated SOCS expression is associated with several pathologies demonstrating the importance of a tight control of SOCS protein expression levels. Enhanced SOCS2 levels for example correlate with some cancers like chronic myeloid leukaemia (Schultheis et al., 2002; Zheng et al., 2006). It will be of special interest to examine whether SOCS2 interference with the tumor suppressor functions of other SOCS proteins is the underlying mechanism.
contributing to oncogenesis. We uncovered in this thesis a novel level of inter-SOCS regulation that is most likely involved in restoring basal cellular responsiveness, adding a further layer of complexity to regulation of cytokine responses. Although the SOCS box of the targeted SOCS seems to be implicated in the interaction with the inhibitory SOCS, the precise nature of this inter-SOCS interaction is still unclear and needs further examination. Detailed analysis of the kinetics of relative expression levels and the binding affinities of all interacting components in a particular cellular background will be required to achieve full and global insights into the precise regulatory control.

Finally, we demonstrated that besides the SH2 domain, the SOCS box can also be involved in substrate recognition by SOCS proteins. Receptor interaction and functionality of CIS were shown to require Elongin B/C recruitment to the SOCS box. This Elongin B/C dependency seems to be an exclusive property of CIS since none of the other examined SOCS members was found to need Elongin recruitment for interaction with target receptor motifs. This is reminiscent of the effects observed for the Y253F mutation in the C-terminal part of the CIS SOCS box as this mutation also completely abrogates functional interaction with most cytokine receptor interaction motifs (Lavens et al., 2007; Uyttendaele et al., 2007). Modelling studies based on the crystal structure of SOCS2 and SOCS4 in complex with Elongin B/C (Bullock et al., 2006; Bullock et al., 2007) predict that the C-terminus of CIS is buried in the interface between the SH2 domain and the SOCS box. Based on this model a direct interaction of the SOCS box or Y253 with the phosphopeptide substrate could be excluded. We propose that the correct positioning of the CIS C-terminus and more specifically of Y253 is essential for substrate binding and that it is induced by structural changes in the SOCS box upon binding of Elongins. Taken together, we report that Elongin B/C association to CIS may control substrate binding via allosteric modulation of the SOCS box. This represents a unique regulatory mechanism by which the SOCS box may form an on/off switch acting on the SH2 domain. Our observation that Elongin B/C association cannot be uncoupled from CIS functions further supports a major contribution for the recruitment of E3 ligase activity in CIS-mediated inhibition. Furthermore, this implicates that the previous observation concerning SOCS2 interference with the interaction of CIS at the Y985 of the LR, can be explained in a dual way. First, SOCS2 that binds Elongin C with higher affinity than CIS may scavenge Elongin B/C complexes from CIS leading to loss of substrate binding. Second, SOCS2 can interact with unbound CIS leading to
degradation of the free CIS pool. The list of multiprotein complexes containing Elongin B/C is growing, suggesting that dependent of the expression, localization and relative binding affinities of all these B/C box containing proteins, a competition for Elongin B/C will occur. Accordingly, the availability of free Elongin B/C complex may determine CIS activity. Perhaps, this Elongin B/C dependency of CIS may function as a ‘safety lock’ that guarantees total termination of signalling as only the CIS molecules that are able to recruit the proteasomal machinery will contribute to inhibition. The remarkable discrepancy observed between the unaltered phenotype of CIS-deficient mice and the severe defects in growth, mammary gland development and immune effects of CIS transgenic mice (Li et al., 2000; Matsumoto et al., 1999) may also be indicative of the high risk of unrestrained CIS activity.

Taken together, our findings further underscore the functional complexity of the SOCS box. The diverse effects of this domain are reviewed in chapter 11 and the SOCS box increasingly appears as an important modulator of cytokine actions. Undoubtedly, knowledge of how SOCS proteins are regulated and/or degraded, may lead to development of new strategies that utilize the properties of SOCS for therapeutic purposes. Further clarification of the nature of SOCS regulation will have to deal with major questions: Given the many receptor complexes that are known to recruit SOCS, why did we only identify few substrates of the ubiquitin E3 ligase activity of each SOCS protein in comparison? Some receptors and JAK2 are reported to be degraded by SOCS but no clear evidence exists concerning other receptor complex components like STATs. Probably, SOCS substrates go beyond the cytokine receptor complex since targets as diverse as other SOCS proteins, TLR adaptors and viral proteins have been reported (Kamio et al., 2004; Mansell et al., 2006; Piessevaux et al., 2006; Tannahill et al., 2005). Further studies are also required to define if the cross-regulatory mechanism functions beyond the SOCS family. At present 210 proteins harboring a SOCS box have been described in the mammalian genome. Possibly they could also be subjected to cross-modulation through SOCS-box dependent degradation or scavenging of Elongin B/C. Of note, the manipulation of SOCS actions might provide potent therapeutic options in the treatment of several disorders. Better understanding of the specific domains or residues that have to be targeted to alter their function and expression may provide a structural basis for SOCS inhibitor design in the development of therapies.
References


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Curriculum Vitae

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Publications


Abstracts and Presentations


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30 April 2008,
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Julie
‘Be the change you want to see in the world’

Mahatma Gandhi