Ghent University
Faculty of Medicine and Health Sciences

On the role of SOCS proteins in leptin signalling

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Thesis submitted to fulfil the requirements for achievement of the grade of PhD in Biomedical Sciences
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2006

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Abbreviations

ACINUS	Apoptotic chromatin-condensation inducer in the nucleus
AD	Highly acidic domain
AgRP	Agouti-related protein
AMPK	AMP-activated protein kinase
α-MSH	α-Melanocyte-stimulating hormone
BBB	Blood-brain-barrier
βc	β-Common
BMI	Body mass index
BRET	Bioluminescence resonance energy transfer
CART	Cocaine-amphetamine-regulated transcript
CD	Cluster of differentiation
CIS	Cytokine inducible SH2-containing protein
CLC	Cardiotrophin-like cytokine
CLF	Cytokine-like factor
CML	Chronic myeloid leukaemia
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CRH	Cytokine receptor homology
CSF	Colony stimulating factor
CT-1	Cardiotrophin-1
Cul	Cullin
db	Mouse diabetes gene: coding for the leptin receptor
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
Epo	Erythropoietin
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
ESS	Extended SH2 subdomain
EVH1	Enabled/VASP homology 1 domain
FERM	Four-point-one, ezrin, radixin, moesin
FNIII	Fibronectin type III

FRET	Fluorescence resonance energy transfer
γc	γ-Common
FVIIa	Factor VIIa
G-CSF	Granulocyte-colony stimulating factor
GDNF	Glial cell line-derived neurotrophic factor
GFR	GDNF family receptor
GH	Growth hormone
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp130	Glycoprotein 130
Grb2	Growth receptor bound protein 2
GTP	Guanosine triphosphate
ICV	Intracerebroventricular
HIF-α	Hypoxia-inducible transcription factor-α
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin Growth Factor 1
IL	Interleukin
ISG15	Interferon stimulated gene product 15
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
JAB	JAK binding protein
JAK	Janus kinase
JH	Janus homology domain
KIR	Kinase inhibitory region
LR	Leptin receptor
LRlo	LR long form, LRb
LRsh	LR short form: LRa, main short isoform of the LR
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAPPIT	Mammalian protein-protein interaction trap
MASPIT	Mammalian small molecule-protein interaction trap
MHC	Major histocompatibility complex
MLR	Mixed-lymphocyte reaction
mRNA	Messenger ribonucleic acid
mSOS	Mammalian son of sevenless

MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NES	Nuclear export signal
NGF	Nerve growth factor
NK	Natural killer
NLS	Nuclear localisation signal
NPY	Neuropeptide Y
ob	Mouse obese gene: coding for leptin
OSM	Oncostatin M
PAC	Peptide affinity chromatography
PAP	Pancreatitis Associated Protein
PCM1	Pericentriolar material 1
PDGF	Platelet-derived growth factor
PIAS	Protein inhibitor of activated STAT
PI-3K	Phosphatidyl inositol-3 kinase
PLC-γ	Phospholipase C-γ
POMC	Pro-opiomelanocortin
PRL	Prolactin
PTP	Phosphotyrosine phosphatase
-R	Receptor
Rbx	RING box protein
RLD	RING-finger-like zinc-binding domain
SAF	Scaffold-attachment factor
SAP	SAFA and SAFB, ACINUS and PIAS domain
SCID	Severe combined immune deficiency
SCF	Stem cell factor
SEAP	Secreted alkaline phosphatase
SH2	Src Homology 2
SHP	SH2 domain containing phosphatase
SOCS	Suppressor of cytokine signalling
Spred	Sprouty-related EVH1 domain-containing proteins
SPR	Sprouty-related cystein-rich domain
SPRY	Sprouty
SSI	STAT-induced STAT-inhibitor
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier

TAD	Transcription activation domain
TAP	Tandem affinity chromatography
TCPTP	T-cell PTP
TF	Tissue factor
TGF	Transforming growth factor
TH	T helper
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tpo	Thrombopoietin
TYK	Tyrosine kinase
Ub	Ubiquitin
UbE	Ubiquitin-dependent endocytosis
UbL	Ubiquitin-like
VASP	Vasodilator-stimulated phosphoprotein
VHL	Von Hippel-Lindau
WT	Wild type

A, Ala	Alanine	M, Met	Methionine
C, Cys	Cysteine	N, Asn	Asparagine
D, Asp	Aspartic acid	P, Pro	Proline
E, Glu	Glutamic acid	Q, Gln	Glutamine
F, Phe	Phenylalanine	R, Arg	Arginine
G, Gly	Glycine	S, Ser	Serine
H, His	Histidine	T, Thr	Threonine
I, Ile	Isoleucine	V, Val	Valine
K, Lys	Lysine	W, Trp	Tryptophan
L, Leu	Leucine	Y, Tyr	Tyrosine

Samenvatting

Leptine is een pleiotroop cytokine dat voornamelijk aangemaakt wordt in wit vetweefsel en in de bloedcirculatie aanwezig is in een hoeveelheid die correleert met de totale massa lichaamsvet. Functionerend als een adipostaat, passeert leptine de bloed-hersenbarrière en informeert de hypothalamus over de status van de energiereserves in het lichaam. Vervolgens worden adaptieve responsen geïnduceerd zodat het lichaamsgewicht constant gehouden wordt.

Bij de meeste obese individuen treffen we een staat van leptineresistentie aan. Deze kan te wijten zijn aan een defect in het transport van leptine doorheen de bloed-hersenbarrière en/of in leptine signaaltransductie in de hypothalamus. Daarnaast speelt leptine een rol in een ganse reeks andere, vaak perifere, fysiologische functies zoals voortplanting, botvorming en het immuunsysteem; het kan ook betrokken zijn in de pathogenese van ondermeer auto-immuunziekten.

Er zijn zes isovormen van de leptinereceptor waarvan enkel deze met een lang cytoplasmatisch deel volwaardige signalisatiecapaciteit bezit. Deze signalisatie gebeurt klassiek via de JAK-STAT cascade, via JAK2 en voornamelijk STAT3. Strikte regulatie van deze cascade is van cruciaal belang; SOCS3 en PTP1B zijn hierbij de best gekarakterizeerde inhiberende regulatoren.

In dit werk gaan we dieper in op de rol van SOCS eiwitten in leptine signalisatie. SOCS eiwitten worden klassiek beschouwd als inhibitoren van cytokine receptorsignalisatie; ze bestaan uit een SH2-domein dat verantwoordelijk is voor associatie met fosfotyrosine-motieven, een N-terminaal preSH2-domein en een C-terminale SOCS-box die geassocieerde eiwitten selecteert voor degradatie door het proteasoom. We identificeerden twee leden van de SOCS familie, CIS en SOCS2, als nieuwe interactiepartners van de leptinereceptor. We bestudeerden de bindingsmodaliteiten van SOCS eiwitten en toonden aan dat in het geval van CIS, maar niet van andere SOCS eiwitten, de SOCS box essentieel is voor associatie met de receptor. Bovendien bewezen we dat onderlinge modulatie tussen de SOCS eiwitten gebaseerd is op directe binding en recrutering van het elongine B/C complex door de SOCS box. Dit laatste wijst op een belangrijke rol voor proteasomale degradatie.

Résumé

La leptine est une cytokine pléiotrope produite principalement dans le tissu adipeux blanc et est présente dans la circulation sanguine en quantités qui corrèlent avec la masse totale de graisse corporelle. Fonctionnant comme un 'adipostat', la leptine passe la barrière hématoméningée et communique le statut des réserves graisseuses du corps à l'hypothalamus.

Chez la plupart des individus obèses on trouve une résistance à la leptine, celle-ci peux être provoqué par un défaut dans le transport à travers la barrière hématoméningée et/ou dans la cascade de signalisation dans l'hypothalamus. Mis à part son importance dans le réglage du poids corporel, la leptine a un rôle dans une série d'autres fonctions physiologiques, souvent périfère comme la reproduction, la formation osseuse et le système immunitaire.

Six isoformes du récepteur de la leptine sont discernés et uniquement la forme avec la partie cytoplasmique complète possède une capacité de signalisation absolue. Cette signalisation est transmise classiquement par la cascade JAK-STAT et JAK2 et STAT3 sont les acteurs principaux. Une régulation rigoureuse de cette cascade est primordiale; SOCS3 et PTP1B sont dans ce cadre là les inhibiteurs le mieux caractérisés.

Dans cette thèse de doctorat nous approfondissons le rôle des protéines SOCS dans la signalisation de la leptine. Les protéines SOCS sont classiquement considérées comme étant des inhibiteurs de signalisation de récepteur de cytokines ; elles contiennent un domaine SH2 qui permet l'association avec des motives de tyrosines phosphorylées, du côté de l' N-terminus un domaine pre-SH2 et vers le C-terminus un domaine dit SOCS-box qui a la capacité de sélectionner des protéines associées pour la dégradation par le protéasome. Nous avons identifié deux membres de la famille SOCS, SOCS2 et CIS, en tant que nouveaux interacteurs du récepteur de la leptine. Nous avons examiné les modalités d'interaction des protéines SOCS et avons pu démontrer que dans le cas de CIS, mais pas des autres protéines SOCS, le 'SOCS-box' est essentiel pour l'association avec le récepteur. De plus, nous avons prouvé que le modulation réciproque entre les protéines SOCS est basé sur une interaction directe et le recrutement du complexe contenant les elongines B et C par le 'SOCS-box'. Cette dernière donnée indique un rôle important pour la dégradation protéasomale.

Summary

Leptin is a pleiotropic cytokine that was initially identified as a key player in food intake and energy expenditure. It is produced mainly in adipose tissue and circulating leptin levels correlate well with the amount of body fat. As an adipostat, leptin passes the blood brain barrier to inform the hypothalamus about the status of energy reserves in the body. Appropriate responses are triggered to maintain a stable body mass. Most obese individuals have developed a state of leptin resistance because their body is not capable of reacting properly to the leptin signal. This leptin insensitivity can be caused by defects at different levels in the leptin pathway including leptin transport through the blood brain barrier, hypothalamic leptin signalling and downstream effects of leptin in the neuronal circuit. In addition, leptin is involved in a broad range of other, often peripheral, physiological functions including reproduction, bone formation and immunity, and may also contribute to the development of disorders like auto-immune diseases.

There are at least six splice variants of the leptin receptor but only the one with a long cytoplasmic tail has full signalling capacities. It typically signals through the JAK-STAT pathway via JAK2 and predominantly STAT3. Since leptin action is of great importance throughout the body, signalling must be under stringent control. SOCS3 and PTP1B are the best characterized mediators of leptin signalling termination.

In this work we focus on the role of SOCS proteins in leptin receptor signalling. SOCS proteins are typically inhibitors of cytokine receptor signalling and consist of an SH2-domain that mediates association with phosphotyrosine motifs, an N-terminal preSH2-domain and a C-terminal SOCS-box that is responsible for targeting associated proteins for proteasomal degradation. We identified two members of the SOCS family, CIS and SOCS2, as new interaction partners of the LR. We studied the binding modus of SOCS proteins and demonstrated that the SOCS-box is essential for receptor interaction of CIS but not of other examined SOCS proteins. In addition, we demonstrated that cross-modulation between SOCS proteins depends on direct interaction and requires elongin B/C recruitment to the SOCS-box which implicates a role for proteasomal degradation.

Part I

General introduction

Chapter 1: Cytokines and their receptors

1. Cytokines

Cytokines constitute a broad group of messenger proteins involved in intercellular communication in multicellular organisms. They regulate biological activities like cell proliferation, differentiation and apoptosis and are involved in many processes including haematopoiesis, homeostasis, modulation of immune responses and development of multicellular organisms.

Two crucial characteristics of cytokines are their redundancy and pleiotropy. Redundancy implies that different cytokines can exert similar biological activities. Upon the failure of one cytokine others can function as a back-up to preserve important cellular functions. Pleiotropy indicates that one particular cytokine can induce multiple biological activities on a number of target cells, thus orchestrating a coordinated response of different cellular processes.

Cytokines have a diverse nomenclature since they were originally named according to their activity or origin. The first cytokines to be described were lymphokines and monokines referring to the cells that produce them, lymphocytes or monocytes. Others are interleukins (IL) that signal between leukocytes during immune responses, interferons (IFN) which are involved in protection against viral infections, growth factors which induce growth and colony-stimulating factors (CSF) that stimulate colony expansion of haematopoietic cells. Since cytokines often show overlapping activities and in many cases are produced by several different cell types this nomenclature is very complex with many cytokines carrying more than one name.

Since cytokines show little sequence homology, they are classified based on structural similarities. Currently, four classes of cytokines are described (Nicola, 1994)

Class I cytokines: 4-α-helical bundles

This large group of cytokines includes the previously mentioned interleukins, interferons and colony stimulating factors. They adopt an anti-parallel 4-α-helical bundle structure with a typical up-up-down-down configuration (figure 1).

These class I cytokines are divided into two subgroups. The first group includes GM-CSF and several interleukins like IL-2, IL-3, IL-4, IL-5. These 'short chain' cytokines have short α-helices and comprise two short antiparallel β-strands in their loops. Leptin, erythropoietin (Epo), prolactin (PRL), leukaemia inhibitory factor (LIF), granulocyte-colony stimulating factor (G-CSF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-6 , IL-11 and growth hormone (GH) are 'long chain' cytokines. They have longer α-helices and the loops contain additional helices.

The IFN/IL-10 family comprises the type I IFNs including IFNα, IFNβ, IFNω, IFNκ and IFNε, the type II IFN (IFNγ), type III IFNs (IFNλ1, IFNλ2 and IFNλ3) and IL-10 with its viral and cellular homologues (Pestka et al., 2004). IFNγ and IL-10 form intermeshed, V-shaped homodimers. Each subunit of the IL-10 dimer consists of six alpha-helices, four originating from one subunit and two from the other, of which four helices have the classical up-up-down-down bundle. The viral IL-10 homologues form dimers whereas cellular homologues which include IL-19, IL-20, IL-22 and IL-24 are monomers (Zdanov, 2004). Several subfamilies can be discriminated within the IFN/IL-10 family based on structure and common receptor use.

Class II cytokines: long chain β-sheet structures

The IL-1 family of cytokines and pro-inflammatory tumour necrosis factor (TNF) related cytokines belong to the Class II cytokines. These cytokines are often produced as membrane-bound precursors and are released from the cell surface by shedding. Their structures are based on β-strands. The TNF family adopts a β-jelly roll fold while the IL-1 related cytokines have a β-trefoil configuration.

Class III cytokines: mosaic structures, type I

This heterogeneous group of cytokines often modulates mitogenic responses. Growth hormones of the epidermal growth factor family (EGF) carry an EGF domain

containing two or more anti-parallel β-strands. Insulin and insulin-related cytokines are characterized by a three α-helical structure linked by three disulfide bridges. This class of cytokines also includes the platelet-derived growth factors (PDGF), nerve growth factors (NGF) and the transforming growth factor β (TGFβ). The latter adopts a 'cysteine knot' configuration in its biologically active form, a disulfide linked dimer with three disulfide bonds in a threaded ring configuration within each monomer.

Class IV cytokines: mosaic structures, type II

The class IV cytokines are a group of very small cytokines, also called chemokines. They have an important role in chemotactic migration of leukocytes. This class of cytokines is further subdivided in four groups depending on the position of the conserved cysteins: CXC, CX3C, CC, C chemokines.

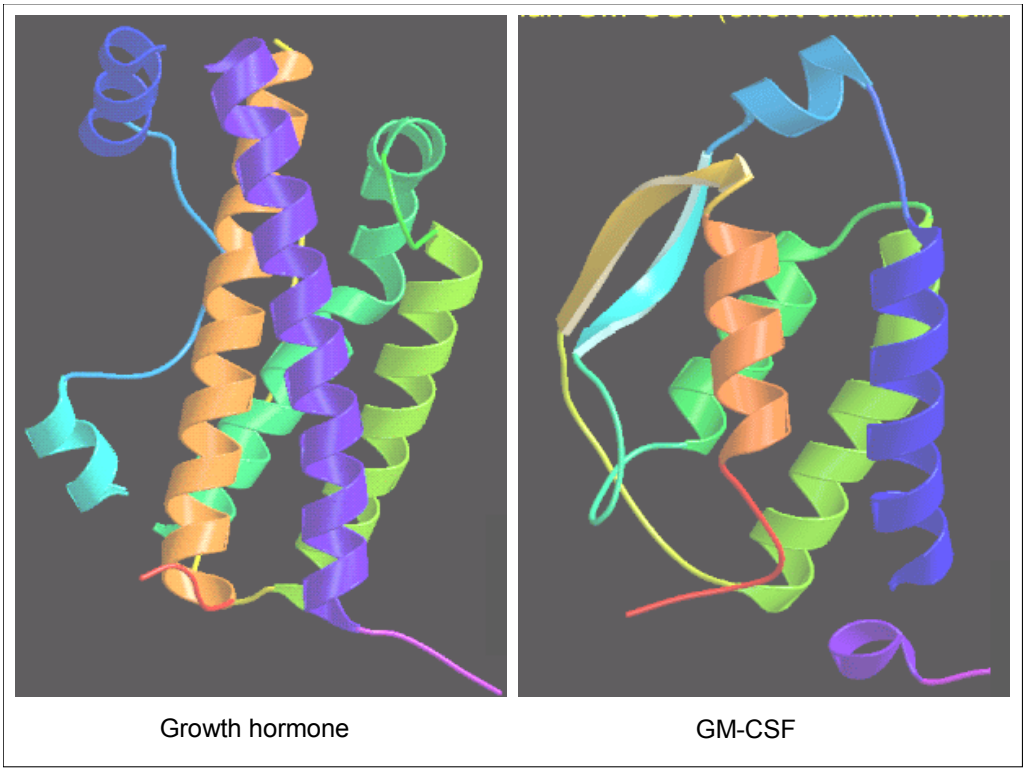

Figure 1: Class I cytokines: 4-α-helical bundles

On the left hand site the structure of growth hormone, a typical long chain cytokine, on the right the structure of GM-CSF, a short chain cytokine.

(Adapted from 'the cytokine web'

url http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/index.html)

2. Cytokine receptors

Since cytokines have no intrinsic enzymatic activity and are incapable of penetrating the cell-surface, they need specific receptors (R) to induce signal transduction and evoke a specific cell response. These cytokine receptors are transmembrane molecules that allow the transfer of the extracellular signal to the cytoplasm. Many cytokine receptors also have a soluble variant consisting of the extracellular part of the membrane bound form. These originate from alternative spliced messenger RNA (mRNA) or from proteolytic release of the extracellular receptor domain (Heaney et al., 1996). Secreted receptors can act as antagonists, as observed for many interleukin receptors. They inhibit cytokine signalling by competing with their membrane-anchored counterparts for the common ligand (Heaney et al., 1996). The soluble receptor can also function as a carrier for the ligand, thereby protecting it from degradation or secretion and therefore significantly prolonging its half-life (Baumann, 1995; Peters et al., 1996). In some cases soluble receptors are part of the receptor complex and facilitate receptor signalling, like seen for the IL-6 receptor complex (Heaney et al., 1996).

Based on their secondary and tertiary structural similarities, cytokine receptors are divided into four categories. In general there is a striking correlation between the structural class of the cytokines and the receptors that they activate.

Class I cytokine receptors: haematopoietin/interferon receptors

Class I cytokine receptors are transmembrane glycoproteins with a characteristic cytokine receptor homology (CRH) domain in their extracellular domain. Each receptor carries at least one of these domains formed by two homologous barrel-like subdomains of about 100 amino acids (figure 2). Both barrels consist of 7 β-strands separated by a proline-rich segment and show great resemblance with the fibronectin type III (FNIII) structures. Other conserved features in the amino acid sequence of the CRH domain are two pairs of cysteines forming disulfide bridges in the first N-terminal domain, a canonical Trp-Ser-X-Trp-Ser (WSXWS) motif in the membrane-proximal subdomain and a stretch of aromatic residues.

The class I receptor family is subdivided into two groups. The type I cytokine receptors are mostly triggered by 4-α-helical bundle class I cytokines while the type II cytokine receptor family binds IFNs and the IL-10 family. The type II cytokine receptors do possess a CRH domain but lack a WSXWS motif. The same type II cytokine receptor complexes are often used by different cytokines for their signal transduction. This is, for example, the case for the type I IFNs including IFNα, IFNβ, IFNω, IFNκ and IFNε that all use the IFNAR-1 and IFNAR-2 receptor and for several subfamilies of the IL-10 family (figure 3) (Langer et al., 2004; Renauld, 2003).
The cytoplasmatic domain of the class I cytokine receptors is more diversified. They have no intrinsic enzymatic activity but associate with intracellular enzymes, JAK kinases, to trigger phosphorylation upon stimulation. The receptor carries two functional domains termed box1 and box2 which are involved in association of Janus kinase (JAK) molecules for signal transduction via the so-called JAK-STAT (signal transducer and activator of transcription) pathway which will be discussed in more detail later on.

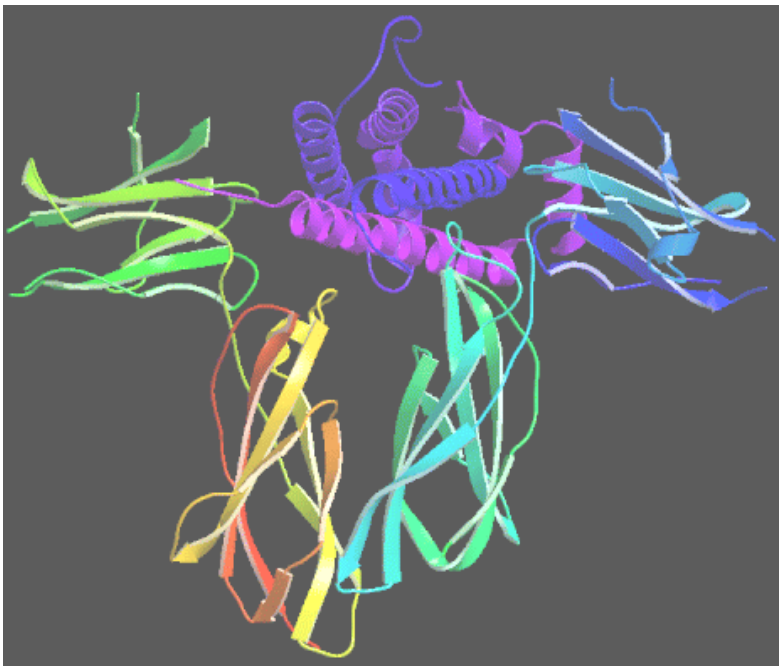

Figure 2: Class I cytokine receptors:
Growth hormone in complex with two extracellular domains of its receptor
(adapted from 'the cytokine web'
url http://cmbi.bjmu.edu.cn/cmbidata/cgf/CGF_Database/cytweb/index.html)

Some class I cytokine receptors are built of one type of subunit and function as a homodimer like the EpoR or the GHR. Others form heteromers sharing one receptor unit and are accordingly grouped into subfamilies. These receptor complexes combine a cytokine-specific unit with a shared signal transducing receptor. Three well characterized type I cytokine receptor families have the glycoprotein 130 (gp130) chain, the β-common (βc) or the γ-common (γc) receptor chain in common. Common use of a cytokine receptor chain in several receptor complexes also occurs for the type II cytokine receptors. This phenomenon of common receptor units partially explains the functional redundancy of cytokines. An overview of the class I cytokine receptor families is shown in figure 3.

Class II cytokine receptors: NGF/TNF and IL-1 receptors

The unifying feature of this receptor class is a domain consisting of six cysteine residues. Each receptor has 4 copies of this domain in its extracellular part. In the cytoplasmatic tail they often carry a so-called 'death domain' that is involved in apoptosis. The IL-1 type receptors have an additional extracellular immunoglobulin (Ig)-like domain.

Class III cytokine receptors: receptor kinases

Unlike the other cytokine receptor classes, class III cytokine receptors have an intrinsic kinase activity. They carry a catalytic domain in the cytoplasmatic tail. The receptor kinases are divided into two subgroups according to substrate specificity, phosphorylating either tyrosine residues (e.g. Insulin R) or serine and threonine residues (e.g. TGFβR).

Class IV cytokine receptors: serpentine receptors

The most remarkable feature of this group is that unlike the other cytokine receptor classes that have a single transmembrane domain, these receptors traverse the membrane seven times. They have a short extracellular domain and the intracellular tail carries several serine and threonine residues that become phosphorylated in receptor signalling. Signal transduction is dependent on additional G-proteins. These are GTP-binding, hetero-trimeric proteins associated with the cytoplasmatic side of the cell membrane. Ligand association induces a conformational change of the receptor which leads to association and activation of the G-protein, that in turn relays the signal to effector enzymes.

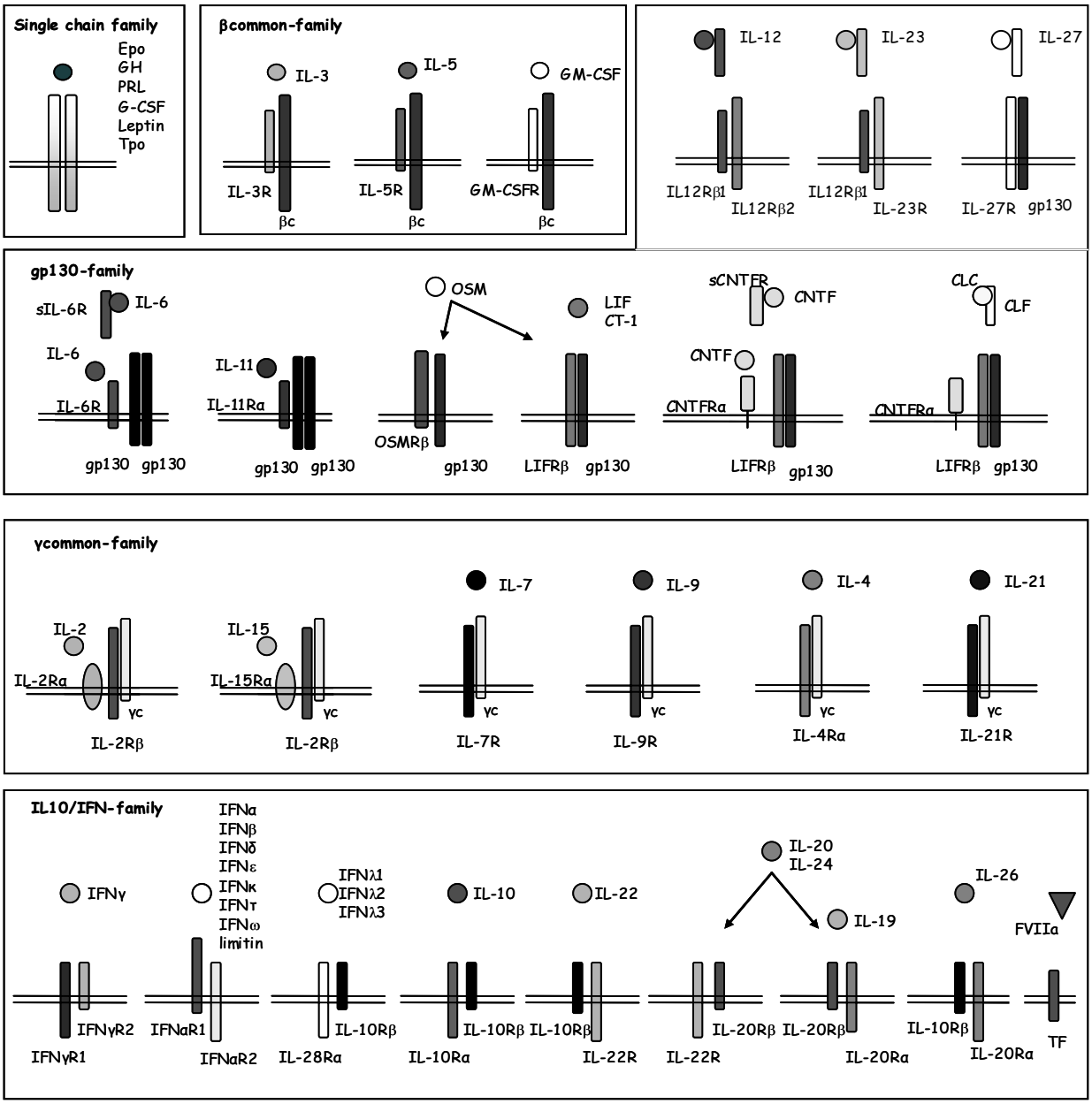

Figure 3: Schematic overview of the Class I cytokine receptors

(adapted from Huising et al., 2006; Ernst and Jenkins, 2004; Renauld, 2003).

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Chapter 2: Cytokine receptor signalling

1. The JAK-STAT pathway

Class I cytokine receptors typically signal trough the JAK-STAT pathway (figure 4). As these cytokine receptors do not possess any intrinsic kinase activity, they have JAK kinases constitutively associated with the cytoplasmatic membrane proximal part of the receptor. Ligand association induces receptor clustering and reorganisation thereby bringing the associated JAK kinases in close proximity and allowing them to activate each other by cross-phosphorylation. These activated JAK kinases then phosphorylate cytoplasmic tyrosines in the receptor tail. These phosphorylated residues serve as docking sites for various signalling molecules. STAT molecules are typically recruited to these phosphotyrosines. Upon recruitment the STATs become also phosphorylated by the JAKs. The activated STATs dissociate from the receptor and translocate to the nucleus as dimers where they induce transcription of specific target genes.

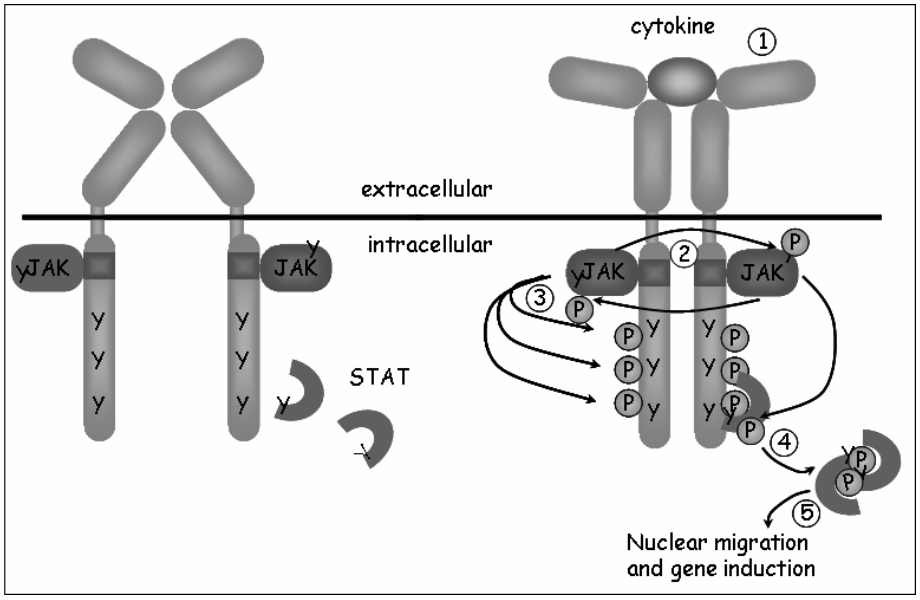

Figure 4: Schematic representation of the JAK-STAT pathway:
Ligand association leads to reorganisation of the receptor chains (step 1) and activation of the juxtaposed JAKs through cross-phosphorylation (step 2). Intracellular tyrosines are subsequently phosphorylated and act as docking sites for STAT molecules (step 3). The STATs are activated by phosphorylation and dissociate from the receptor (step 4). They form dimers that translocate to the nucleus to induce specific target genes (step 5).

Receptor activation

The initial step in receptor activation is induced by binding of the cytokine. Upon ligand association the receptor chains are reorganized. Two models have been proposed for cytokine induced receptor oligomerization. A first model describes the individual receptor chains as monomers on the cell membrane. Upon cytokine administration one receptor chain associates with the ligand and this induces recruitment of one or more receptor chains resulting in the formation of an activated receptor complex. This mechanism of receptor clustering was first proposed for the GHR (Cunningham et al., 1991; Wells et al., 1994).

In the second model the cytokine receptor chains are pre-assembled at the membrane. Cytokine association induces a conformational change which is allosterically transduced to the intracellular part of the receptor. The membrane proximal cytoplasmic regions of the receptors are brought in closer proximity which results in juxtaposing and subsequently activating the associated JAK kinases. This model was for example supported by the crystal structures described for the EpoR complex with or without its ligand (Livnah et al., 1999; Remy et al., 1999). The transmembrane domain of the EpoR is involved in its ligand-independent dimerization (Constantinescu et al., 2001).

The first model proposed was previously generally accepted. However, preclustering has since been suggested for a variety of class I cytokine receptors including the GHR (Frank et al., 2002; Ross et al., 2001). From the different receptor systems examined it is clear that receptor activation is a complex system that is not yet fully understood.

The JAKs

As mentioned before class I cytokine receptors have no intrinsic kinase activity. The tyrosine-specific phosphorylation that occurs upon ligand binding and subsequent receptor oligomerisation is mediated by the activity of cytoplasmic tyrosine kinases. These kinases are called JAKs, 'Just Another Kinase' or 'JAnus Kinase' named after Janus, the Roman god of gates and doors (ianua), beginnings and endings, and hence represented with a double-faced head, each looking in an opposite direction,

referring to the unique feature of JAK molecules: their kinase and pseudokinase domain.

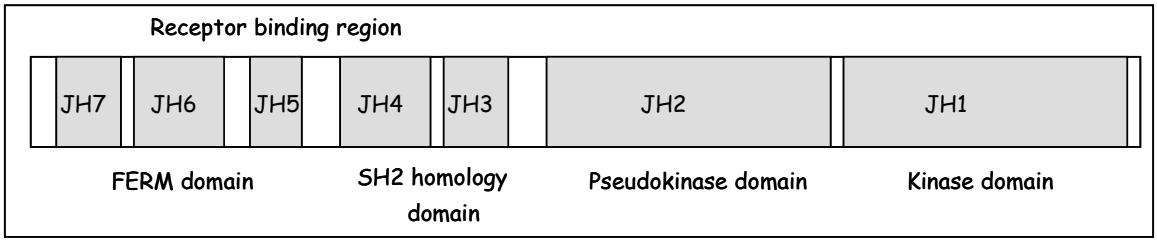

Figure 5: Schematic representation of the JAK structure

JAKs are rather large proteins of about 1100 amino acids. All JAK kinases have seven regions of high homology called Janus homology domains 1 to 7 (JH1-JH7), which are numbered starting from the C-terminus (figure 5). The N-terminal domains (JH7-JH5) are most likely involved in receptor association and form a structure that shares similarity with a FERM (Four-point-one, Ezrin, Radixin, Moesin) domain, which is known to be involved in interactions between proteins (Girault et al., 1998). This domain mediates receptor binding and plays a role in the preservation of the catalytical activity (Hilkens et al., 2001; Zhou et al., 2001). The JH4-JH3 regions form a structural domain resembling a Src Homology 2 (SH2) domain, but appear not to be involved in mediating phosphotyrosine dependent protein interactions, as SH2 domains typically do. However, the JH4-JH3 region is structurally important for receptor association and receptor surface expression (Radtke et al., 2005). As mentioned above JAKs have two kinase domains in their C-terminal part, a functional kinase domain JH1 preceded N-terminally by a pseudokinase domain JH2. This kinase-like domain has no enzymatic activity although it has all the structural characteristics of a normal tyrosine kinase domain. However, it is important for a normal catalytic activity of the JAK kinase. Deletion of the pseudokinase domain results in the dysregulation of the JAK2 kinase activity and a loss of function for TYK2 (Saharinen et al., 2000; Velazquez et al., 1995; Yeh et al., 2000). A structure model for JAK2 is shown in figure 6.

The JAK family comprises four mammalian members, JAK1, JAK2, JAK3 and TYK2. Different JAKs are involved in signalling via different cytokine systems and some cytokine receptors even activate more than one kinase. JAK1, JAK2 and TYK2 are ubiquitously expressed whereas JAK3 expression is restricted mainly to haematopoietic cells (Leonard and O'Shea, 1998). Cytokine receptors can be categorized into subfamilies according to the JAKs and STATs they use for signalling, which is outlined in table 1.

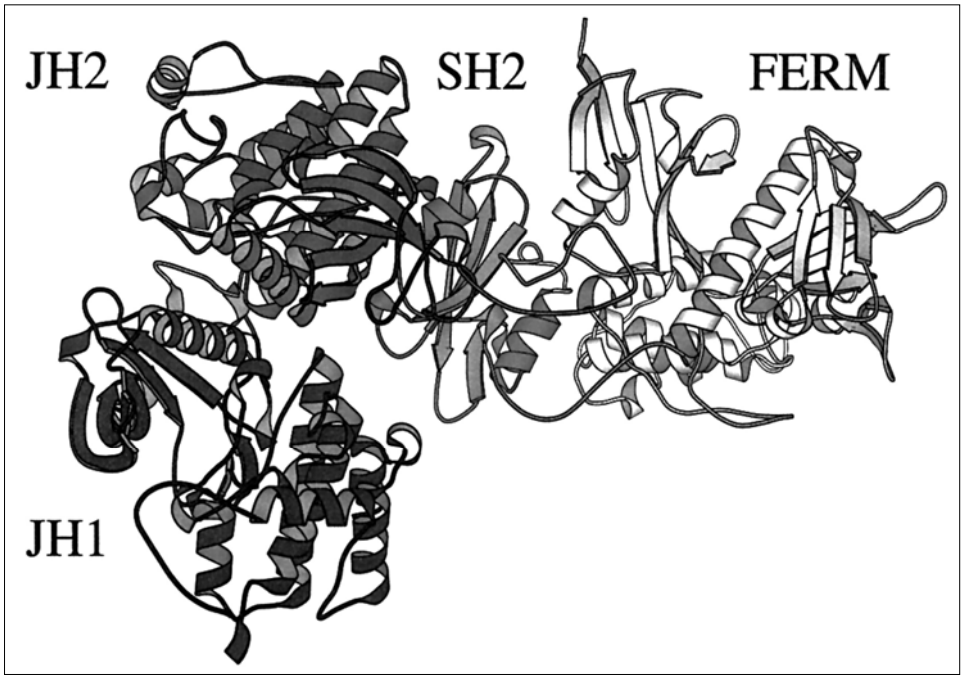

Figure 6: JAK2 structure model

(from Giordanetto and Kroemer, 2002).

It appears that the role of JAKs is more than mere tyrosine phosphorylation. JAK2, for example, associates with the EpoR in the endoplasmatic reticulum (ER) and this appears essential for membrane expression of the receptor (Bonifacino et al., 2002; Huang et al., 2001). Likewise, JAK1 is involved in cell surface expression of the OSM receptor. TYK2 is essential for stable membrane expression of a subunit of the type I IFN receptor complex (Ragimbeau et al., 2003). In addition, JAK1 is also implicated in translocation of STAT1 to the nucleus (Mowen and David, 2000). The phosphotyrosines in activated JAK kinases also function as docking sites for several proteins including the SH2-B homologues (O'Shea et al., 2002; Rui et al., 2000).

Table 1: Cytokine specific JAK and STAT activation

(adapted from Schindler and Strehlow, 2000)

ligands	*JAK kinases*	*STATs*
Single chain family		
Epo	JAK2	STAT5
GH	JAK2	STAT5, (STAT3)
PRL	JAK2	STAT5
G-CSF	JAK1	STAT3
Leptin	JAK2	STAT3, (STAT1, STAT5)
Tpo	JAK2	STAT3, STAT5
β_c-family		
IL-3	JAK2	STAT5
IL-5	JAK2	STAT5
GM-CSF	JAK2	STAT5
γ_c-family		
IL-2	JAK1, JAK3	STAT5, (STAT3)
IL-4	JAK1, JAK3	STAT6
IL-7	JAK1, JAK3	STAT5, (STAT3)
IL-9	JAK1, JAK3	STAT5, STAT3, STAT1
IL-15	JAK1, JAK3	STAT5, (STAT3)
gp130 family		
IL-6	JAK1, JAK2	STAT3, STAT1
IL-11	JAK1	STAT3, STAT1
OSM	JAK1, JAK2	STAT3, STAT1
LIF	JAK1, JAK2	STAT3, STAT1
CNTF	JAK1, JAK2	STAT3, STAT1
IFN/IL-10 family		
IFN-α/β	TYK2, JAK1	STAT1, STAT2
IFN-γ	JAK1, JAK2	STAT1, (STAT5)
IL-10	TYK2, JAK1	STAT3
IFN-λ1-3	JAK1, TYK2	STAT1, STAT2, STAT3, STAT4, STAT5
IL-19	JAK1	STAT1, STAT3
IL-20	JAK1	STAT1, STAT3
IL-22	JAK1, TYK2	STAT1, STAT3, STAT5
IL-24	JAK1	STAT1, STAT3
IL-26	JAK1, TYK2	STAT3, STAT1

Biological functions – JAK knock-outs

Analysis of knock-out mice is crucial for deciphering the functions of JAK kinases. An overview of the phenotypes of JAK knock-out mice is shown in table 2.

JAK1 knock-out mice are small and die early after birth. They exhibit neurological defects that cause problems with suckling. JAK1 deficiency affects signalling of IFNs and cytokines that use the γ_C-chain or the gp130 chain (Rodig et al., 1998).

JAK2 deficiency causes embryonic lethality due to the absence of a definitive erythropoiesis. $JAK2^{-/-}$ fibroblast cells are clearly impaired in IFNγ signalling (Neubauer et al., 1998; Parganas et al., 1998).

Targeted disruption of the murine JAK3 gene results in a viable phenotype. However these mice suffer from severe combined immune deficiency (SCID). $JAK3^{-/-}$ mice show profound defects in T- and B-cells due to impaired signalling via the γ_c receptors. In contrast, although JAK3 deficient humans have severe T-cell defects and SCID, their B-cell populations remain unaffected (Thomis and Berg, 1997).

TYK2-deficient mice show defective responses to IL-12 and lipopolysaccharide (LPS). Surprisingly, only subtle defects were observed in IFNα and β signalling (Shimoda et al., 2000).

The STATs

The family of STATs consists of seven mammalian members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. They map in three gene clusters with each cluster mapping to a different chromosome. STAT1 and STAT4 are located on chromosome 1, STAT2 and STAT6 are found on chromosome 10 and STAT3, STAT5a and STAT5b map to chromosome 11. STAT5a and STAT5b are over 95% homologous, which results from a very recent additional duplication of the STAT5 gene (Copeland et al., 1995; Mui et al., 1995).

STAT isoforms have also been detected. They result from protein processing as found for STAT5a, STAT5b and STAT6 (Azam et al., 1997;Quelle et al., 1995; Wang

et al., 1996) or alternative splicing of the mRNA including STAT1β, STAT3β, STAT5a1 and STAT5a2 (Kazansky et al., 1995; Schaefer et al., 1995; Shuai et al., 1993).

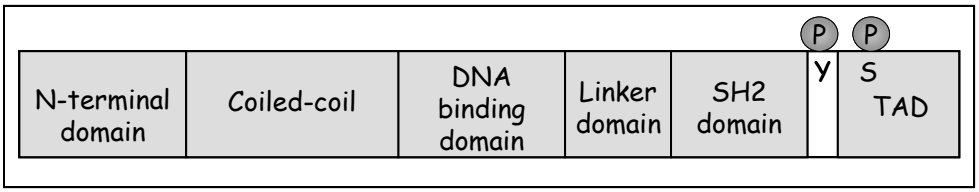

Figure 7: Schematic representation of the STAT structure

STATs are about 800 amino acids long and have six structurally and functionally conserved domains (see figure 7).

In the N-terminal region, STAT molecules have a coiled-coil domain, preceded by a conserved dimer-dimer interaction domain. The coiled-coil structure consists of four helices and is responsible for interactions with other transcription factors including c-Jun and NFκB (Ortmann et al., 2000; Schaefer et al., 1995; Shen et al., 1998). In addition, the domain is also important for receptor binding and nuclear translocation (Begitt et al., 2000; Ma et al., 2003; Zhang et al., 2000). The extreme N-terminal dimer-dimer interaction domain apparently allows STAT dimers to form tetramers that cooperatively associate with multiple STAT binding sites in the promoter sequence (John et al., 1999; Vinkemeier et al., 1996; Xu et al., 1996; Murphy et al., 2000).

The central part of STAT proteins carries a DNA binding domain, a linker domain, an SH2 domain and a conserved tyrsosine phosphorylation site. The DNA binding domain is a β-barrel with an Ig-fold and resembles the structure of the p53 and NF-κB DNA binding domains. Obviously, it is implicated in DNA binding, however, there are only little direct interaction sites (Becker et al., 1998; Chen et al., 1998). Figure 8 represents the nutcracker-like structure that is formed by the association of STAT1 dimers with DNA. The SH2 domain is essential for receptor association and dimerization of the STATs. Its pocket structure consists of a β-sheet flanked by two α helices. A conserved arginine in this pocket is essential for association of phosphotyrosines (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 activated STATs is based on the reciprocal interaction of the SH2 domain with this phosphorylated tyrosine (see figure 8). STAT association with the JAKs may also be attributed to the SH2 domain (O'Shea et al., 2002).

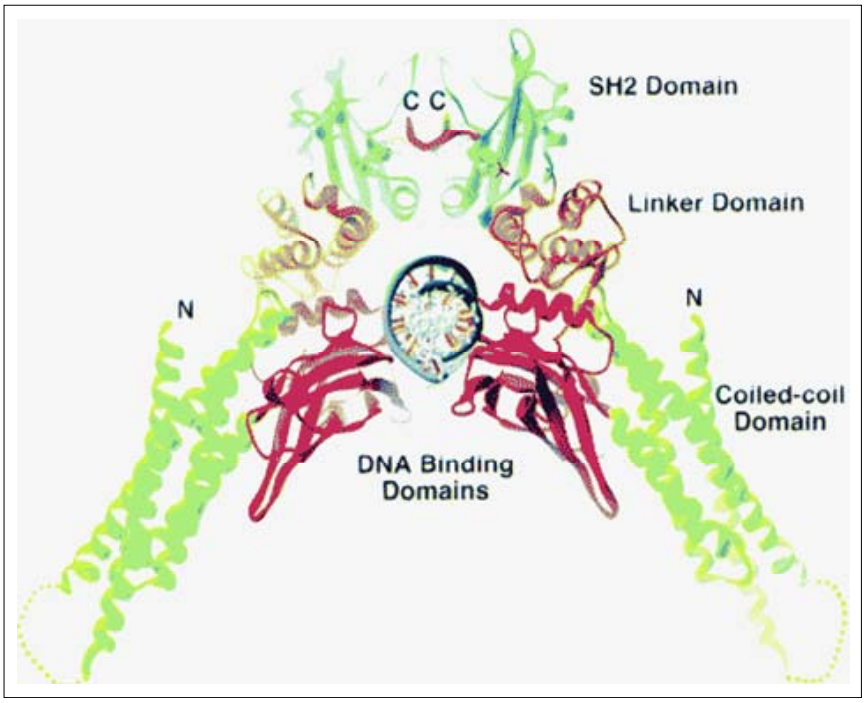

Figure 8: Structure of STAT1 in complex with DNA
(adapted from Chen et al, 1998).

The C-terminus of STATs consists of a variable transcription activation domain (TAD). Its poor conservation among various STATs can be explained by its regulatory function in unique transcriptional responses. In many cases phosphorylation of the conserved serine within this domain leads to an enhanced transcriptional activity and may be involved in the TAD dependent regulation of transcription (Decker and Kovarik, 2000; Kovarik et al, 2001).

STAT molecules are recruited to the phosphotyrosines of activated receptors and after becoming phosphorylated themselves at their conserved tyrosine by the JAKs they dissociate from the receptor and dimerize. STAT1, STAT3 and STAT4 can form both homo-and heterodimers whereas for STAT5 and STAT6 only homodimers have

been demonstrated. STAT2 is only functional after dimerization with STAT1 or STAT4.

When STAT dimers reach the nucleus they associate with specific sequences in the promoter of target genes. These binding consensus sequences are typically palindromes: STAT1, STAT3, STAT4, STAT5a, STAT5b and STAT6 bind to a 5'-TTCN_{2-4}GAA-3' motif, while the STAT1-STAT2 dimer binds to an IFN-stimulated response element with the consensus sequence AGTTTN_3TTTCC (Kisseleva et al., 2002).

STAT proteins have no classical nuclear localisation signal (NLS) and the mechanisms that regulate their exchange between the cytoplasm and the nucleus are not really clear. Translocation of STAT dimers to the nucleus involves an active nuclear import mechanism depending on Ran and importin-α5. A nuclear export signal (NES) in the DNA binding domain is likely to drive the nuclear export of the STATs mediated by Ran and exportin 1. STAT dephosphorylation and dissociation from the DNA uncovers this NES sequence and activates the export mechanism mediated by exportin1 (Kisseleva et al., 2002; McBride and Reich, 2004; O'Shea et al., 2002).

Table 2: Phenotype of mice deficient in various JAKs and STATs

Knock-out	*Phenotype*
JAKs	
JAK1	Early postnatal lethality, neurological deficiencies, SCID
JAK2	Embryonic lethality, impaired erythropoiesis
JAK3	Viable, fertile, SCID
TYK2	Viable, fertile, defect in antiviral response
STATs	
STAT1	Viable, defect in antiviral response, impaired growth control
STAT2	Viable, defect in antiviral response
STAT3	Embryonic lethal
STAT4	Viable, defective T_H1 differentiation
STAT5a	Viable, impaired breast tissue development
STAT5b	Viable, impaired growth
STAT6	Viable, defective T_H2 differentiation

Biological functions – STAT knock-outs

Specific disruption of each of the STAT genes revealed distinctive functions for the various STATs. An overview of the different knock-out mice phenotypes is given in table 2.

The primary role of STAT1 in IFN signalling was clearly established by the generation of STAT1 knock-out mice. These mice are highly susceptible to bacterial and viral infections (Meraz et al., 1996). Involvement of STAT1 in non-immune responses like IFN-γ-induced growth retardation was readily reflected in the enhanced tumor susceptibility of $STAT1^{-/-}$ mice (Shankaran et al., 2001). All major defects could be attributed to IFN signalling (Meraz et al., 1996).

STAT2 deficient mice are also defective in viral responses. They are primarily defective in IFNα/β signalling that is mediated by STAT1/STAT2 heterodimers (Park et al., 2000).

STAT3-deficient mice die embryologically before gastrulation. Clearly STAT3 has an essential role in development of various cell lineages early in embryogenesis (Takeda et al., 1997). STAT3-depleted zebrafish embryos die later in embryogenesis but display abnormal cell movement during gastrulation (Yamashita et al., 2002). In order to clarify the specific roles of STAT3, several tissue-restricted mice knock-outs were developed. STAT3 deficient T-cells lose their proliferative response to IL-6 (Takeda et al., 1998). STAT3 deficiency in macrophages and neutrophils leads to high susceptibility to endotoxin shock. Various stimuli like LPS cause these cells to produce more inflammatory cytokines due to impaired IL-10 responsiveness (Takeda et al., 1999). Finally, lack of STAT3 in keratinocytes results in defective wound healing and hair growth (Sano et al., 1999).

The phenotype of STAT4 knock-out mice revealed that STAT4 is essential in IL-12 signalling. A marked tendency towards T helper 2 (T_H2) responses is observed in these mice. Lymphocytes are impaired in T helper 1 (T_H1) differentiation and fail to produce IFNγ in response to IL-12 (Kaplan et al., 1996b; Thierfelder et al., 1996).

STAT4-deficient mice are therefore protected against T_H1 based autoimmune diseases like experimental autoimmune encephalomyelitis (EAE) (Chitnis et al., 2001).

STAT5a and STAT5b are 96% identical in their amino acid sequence. However, their biological functions are not completely redundant, which is underscored by the striking differences in phenotype of the single knock-out mice. STAT5a deficiency predominantly causes impaired PRL-dependent mammary gland development (Liu et al., 1997), whereas STAT5b deficient mice show aberrations in sexual dimorphic growth (Udy et al., 1997). STAT5a/STAT5b double knock-outs were created to assess their functional redundancy and exhibited a more severe phenotype. Many of these mice die within weeks after birth. The surviving mice are smaller and infertile with mammary gland defects. They have relatively normal peripheral blood counts. However, defects in myeloid and lymphoid lineages were observed under stress conditions and NK cells were absent (Teglund et al., 1998).

STAT6 is broadly expressed and is activated by IL-4 and IL-13. STAT6-deficient mice failed to develop T_H2 cells in response to these cytokines. These mice also lose the ability to class switch antibodies to the IgE isotype upon IL-4 stimulation (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996a; Takeda et al., 1996b). Lack of STAT6 significantly attenuates the T_H2 related symptoms in asthmatic disease (Akimoto et al., 1998; Kuperman et al., 1998; Miller et al., 1998).

2. Other cytokine-induced signalling pathways

Cytokine receptors predominantly signal via the JAK-STAT pathway as explained above. However other pathways are also involved in transduction of the cytokine signal. A number of adaptor molecules can associate with the receptor or the kinase and sometimes upon JAK-dependent phosphorylation, link to other signalling pathways.

Here we only describe the well characterized MAPK (mitogen activated protein kinase) pathway (Dong et al, 2002; Platanias, 2002). Other signalling molecules recruited to cytokine receptors include Vav, phospholipase C-γ (PLC-γ) and

phosphatidylinositol-3 kinase (PI-3K) (Carpenter and Ji, 1999; Fresno Vara et al., 2004; Hennessy et al., 2005; Krasilnikov, 2000; Uddin and platanias, 2004).

The Ras-Raf pathway plays an important role in cytokine signal transduction. Extracellular-signal-regulated kinase (ERK) activation can be initiated by Grb2 (growth receptor bound protein 2) association with phosphorylated tyrosine motifs in the cytokine receptor or with phosphorylated adaptor molecules including SH2 domain containing phosphatase-2 (SHP-2), Shc and IRS (insulin receptor substrate)-1/2. Grb2 recruits mSOS (mammalian son of sevenless) to the receptorcomplex. mSOS in turn associates with the membrane-anchored Ras and induces its activation. Ras then triggers the MAPK pathway by activation of Raf, a serine-threonine kinase (Kerkhoff et al., 2001).

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Chapter 3: Negative regulation of cytokine receptor signalling

Typically, the activity of cytokines, involved in a broad range of processes, is very short-lived. Therefore their signalling modus has to be under very stringent control. There are a number of mechanisms that modulate cytokine receptor signalling. Obviously, any impairment or degradation of the key signalling components of the JAK-STAT pathway will attenuate signalling. In addition, several molecules function as active negative regulators of cytokine receptor signalling. The main ones are phosphatases (PTP), protein inhibitor of activated STAT (PIAS) proteins, the suppressors of cytokine signalling (SOCS) family and the recently identified Sprouty-related proteins, the Spred proteins.

1. Internalisation and degradation

Internalisation and degradation of the activated cytokine receptor is an effective mechanism to irreversibly turn off cytokine signalling. The internalisation of many cytokine receptors has been described. A di-leucine motif in the cytoplasmatic tail is often found to be involved in receptor internalisation. Together with an upstream serine it is essential for internalisation of the gp130 chain and down-regulation of the IL-6R, most likely via the clathrin-coated pit pathway leading to lysosomal degradation (Dittrich et al., 1994; Dittrich et al., 1996; Gibson et al., 2000). LIFR internalisation occurs independently of the gp130 chain. The LIFR carries its own di-leucine based internalisation motif with an isoleucine in the second position, in its cytoplasmatic tail (Thiel et al., 1999). A role for a di-leucine internalisation motif in receptor internalisation was also reported for the G-CSFR (Aarts et al., 2004). Proteasome inhibitors prolong the signalling capacity of cytokine receptors. Endocytosis of the GHR is regulated by the ubiquitin (Ub) conjugation system and requires the Ub dependent endocytosis (UbE) motif in its intracellular domain (Govers et al., 1999). Ub also plays a role in internalisation of the EpoR. The cytokine inducible SH2 domain containing protein (CIS) is involved in recruitment of the proteasome and may target the EpoR for degradation (Verdier et al., 1998). CIS is a member of the family of SOCS proteins that is discussed below. Degradation of the EpoR was also addressed to lysosome activity. It was suggested that a significant part of the intracellular domain of the EpoR is targeted to the proteasome, while the remaining part of the receptor complex is degraded in the lysosome (Walrafen et al., 2005). Ubiquitination is described in the text box on ubiquitination below.

Text Box: ubiquitination

Ubiquitin (Ub) is a small, 76 amino acid long protein that is highly conserved among eukaryotic organisms. Ubiquitination is the process of its covalent conjugation to other proteins. Ub modification can influence the stability, activity or subcellular location of the target protein.

Ubiquitination is a multistep mechanism that involves three types of enzymes. The Ub-activating enzyme E1 activates Ub by forming a thioester bond. Activated Ub is subsequently transferred to an Ub-carrier enzyme E2 by transthiolation. Finally, an Ub ligase E3 conjugates ubiquitin to a lysine residue or the N-terminus of the E3 associated target protein (Glickman and Ciechanover, 2002; Breitschopf et al., 1998). The specific association of the E3 ligases with their target proteins determines substrate selection for ubiquitination. Ubiquitination is a reversible and dynamic process. It is controlled by de-ubiquitinating enzymes that cleave off Ub (Weissman, 2001).

Proteins can be modified by a single or by multiple Ub moieties, termed mono-, multi- or polyubiquitination. Poly-Ub chains are formed by conjugation of Ub to any of the seven lysines in the previously conjugated Ub. The different types of ubiquitination are thought to target the associated protein for different cellular events. Mono-ubiquitination for instance is involved in histone regulation and receptor internalization (Belouzard et al., 2006; Osley, 2004; Wilkinson et al., 2005). In many cases polyubiquitination leads to protein degradation 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). Lysine48-linked poly-Ub chains usually target proteins for proteasomal degradation (Kim and Rao, 2006; Pickart, 2001; Thrower et al., 2000).

A well-studied example is the Von Hippel-Lindau (VHL) tumor suppressor which is involved in the transfer of polyubiquitin chains to hypoxia-inducible transcription factor-α (HIF-α). This results in the degradation of HIF-α by the proteasome and downmodulation of hypoxia-inducible gene expression (Iliopoulos et al., 1996; Pause et al., 1997; Lisztwan et al., 1999; Hon et al., 2002). This phenomenon is regulated by oxidation status and VHL specifically associates with hydroxilated HIF-α in non-hypoxic conditions (Hon et al., 2002; Wilkinson et al., 2005). VHL protein is part of poly-protein complex that functions as a multisubunit E3 protein ligase. It has a SOCS-box like domain that associates with elongin B and C (Duan et al., 1995). Elongin C is responsible for association with the BC-box in the N-terminal part of the SOCS-box while ElonginB binds with elongin C (Stebbins et al., 1999). Cullin2 (Cul2) and the E3 ligase enzyme Rbx1 (RING box protein 1) are recruited to the elongin B/C complex and the Cul2-box in the SOCS-box of VHL (Kamura et al., 2004). Together these proteins form an E3 ubiquitin ligase complex and specifically target associated proteins for poly-ubiquitination and subsequent degradation by the 26S proteasome. Many protein families, including the SOCS protein family that is discussed later, contain a SOCS-box region and may link a broad range of proteins to the common process of proteasomal protein degradation (Hilton et al., 1998; Zhang et al., 1999).

Besides Ub, several other Ub-like (UbL) proteins can also modify target proteins using similar enzymatic mechanisms for their covalent conjugation. These UbL proteins include ISG15 (Interferon stimulated gene product 15), that resembles an Ub dimer and influences signal transduction of IFN or other inflammatory signals, Nedd8 that can influence transcription and SUMO (Small Ub-related modifier), which sometimes acts as an antagonist to ubiquitination (Dohmen, 2004; Ritchie and Zhang, 2004). Ub-like domains with a characteristic Ub fold were also found as stable regions within other proteins, as demonstrated for elongin B (Stebbins et al., 1999). These domains do not become conjugated to other proteins but probably function in Ub-mediated processes (Pickart and Eddins, 2004; Weissman, 2001).

Proteasome inhibitors also stabilize receptor-recruited STAT molecules, as seen for STAT1 in IFNγ signalling. The STATs are a target for ubiquitination and subsequent degradation by the proteasome (Kim and Maniatis, 1996; Wang et al, 2000).
A similar phenomenon was observed for JAK kinases. Proteasome inhibitors prolonged the IL-2 and IL-3 induced activation of the JAK kinases (Callus and Mathey-Prevot, 1998; Yu and Burakoff, 1997).

2. Phosphatase activity

Since tyrosine phosphorylation is a crucial step in the activation of the cytokine signalling pathway, dephosphorylation by PTPs is an obvious factor in negative regulation.
SHP-1 and SHP-2 (SH2 domain containing phosphatase 1 and 2) are two constitutively expressed phosphatases responsible for the dephosphorylation of JAK kinases and consequent inhibition of their activity. Both phosphatases carry two tandem SH2 domains followed by a tyrosine phosphatase catalytic domain (figure 9). They bind to the phosphotyrosine residues of a number of cytokine receptors with their SH2 domains. Interference with SHP-1 recruitment to the EpoR clearly results in prolonged signalling (Klingmuller et al., 1995). Interaction with JAK was also demonstrated for SHP-2 (Yin et al., 1997). While SHP-2 is broadly expressed, SHP-1 expression is restricted to haematopoietic cells (Ahmad et al., 1993; Yi et al., 1992). The phenotype of a natural SHP-1 loss-of function mutant, the motheaten mouse, underscores the critical role of SHP-1 in hematopoiesis and immunity. These mice show severe dysregulations in macrophages and neutrophils which result in skin abnormalities and airway inflammation, and die shortly after birth (Kamata et al., 2003; Shultz et al., 1993; Shultz et al., 1997; Tsui et al., 1993). SHP-2 deficient mice die embryonically probably because of defective EGF signalling (Saxton et al., 1997; Qu et al., 1999). Despite its clear phosphatase activity, accumulating data also demonstrate an activating role for SHP-2. IFN-induced STAT activation is significantly increased in SHP-2 $^{-/-}$ fibroblasts (You et al., 1999). Negative regulation of leptin receptor (LR) and gp130 signalling was also suggested, although this effect may also be attributed to inhibition by SOCS3, a member of the SOCS protein family that is discussed below (Banks et al., 2000; Carpenter et al., 1998; Kim et al., 1998; Lehmann et al., 2003; Nicholson et al., 2000). Conversely, implementation of SHP-2

in the activation of the MAPK pathway was reported for several cytokine systems including leptin, Epo, EGF, IL-2 and insulin (Barber et al., 1997; Bennett et al., 1996; Bjorbaek et al., 2001; Lundin et al., 2002; Milarski et al., 1994; Tauchi et al., 1995). SHP-2 itself becomes phosphorylated and leads to recruitment of Grb2 and subsequent activation of the ERK pathway (Bennett et al., 1994; Lu et al., 2001).
SHP-2 also positively influences signalling by interacting with STAT molecules. SHP-2 maintains tyrosyl phosphorylation of STAT5a and translocates to the nucleus as a complex upon PRL stimulation (Chughtai et al., 2002). On the other hand is SHP-2 also responsible for tyrosine dephosphorylation of STAT5 in the cytosolic compartment (Yu et al., 2000).

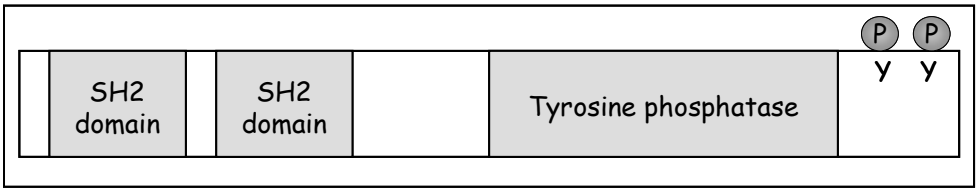

Figure 9: Structure of SHP

The catalytic activity of SHP-2 is firmly auto-regulated by its two SH2 domains. The N-terminal SH2 domain acts as a conformational switch. It either binds the catalytic phosphatase domain and directly inhibits its activity or it interacts with the phosphotyrosine substrate thereby activating the enzyme. Introduction of phosphonates, nonhydrolyzable phosphotyrosine analogues, revealed a role for the C-terminal tyrosines (figure 9). While the N-terminal SH2 domain associates with the N-terminal phosphotyrosine and reduces basal inhibition of the enzymatic activity, the C-terminal SH2 domain interacts with the C-terminal phosphotyrosine and stimulates phosphatase activity (Hof et al., 1998; Lu et al 2001).

Other phosphatases implicated in tyrosyl dephosphorylation are CD45, PTP1B and PTPεC. CD45 is a transmembrane phosphatase that is highly expressed in haematopoietic cells. It negatively regulates IL-3 and Epo signalling by direct dephosphorylation of the JAKs (Irie-Sasaki et al., 2001). PTPεC dephosphorylates JAK kinases and suppresses IL-6 and LIF signalling (Tanuma et al., 2000). The ER-

anchored PTP1B associates with TYK2 and JAK2 and is a key negative regulator of both insulin and leptin signalling (Elchebly et al., 1999; Myers et al., 2001). PTP1B was demonstrated to also dephosphorylate STAT3 (Lund et al., 2005)

Signalling can also be inhibited by dephosphorylation and inactivation of the STAT dimers in the nucleus. TC45 is the nuclear isoform of the T-cell PTP (TCPTP) which is associated with the ER (Lorenzen et al., 1995). It is responsible for the dephosphorylation of STAT1 in the nucleus (ten Hoeve et al., 2002).

3. PIAS

The PIAS protein family consists of four members, PIAS1, PIASx (also known as PIAS2), PIAS3, PIASy (also known as PIAS4) (Shuai and Liu, 2003). Two isoforms were identified for each PIAS protein except for PIAS1. PIAS proteins have an N-terminal nuclear receptor interaction motif and a central zinc-binding domain (figure 10) (Shuai and Liu, 2005).

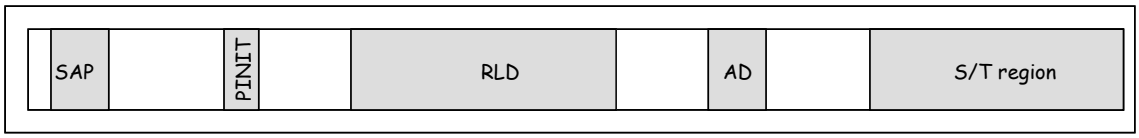

Figure 10: Structure of PIAS

PIAS consist of five conserved domains: The N-terminal SAP domain (scaffold-attachment factor A (SAFA) and SAFB, apoptotic chromatin-condensation inducer in the nucleus (ACINUS) and PIAS domain) with a LXXLL signature motif, the conserved PINIT amino acid motif involved in nuclear retention, the RLD (RING-finger-like zinc-binding) domain, the AD (highly acidic) domain with a SUMO1 (small ubiquitin-like modifier 1)-interaction motif and finally a C-terminal S/T (serine- and threonine-rich) region (adapted from Shuai and Liu, 2005).

PIAS proteins were initially discovered as negative regulators of STAT signalling. PIAS1 was identified as a specific inhibitor of STAT1 (Liu et al., 1998). PIAS1 and PIAS3 associate with STAT1 and STAT3 respectively, and inhibit their activity by interfering with DNA binding (Chung et al., 1997; Liao et al., 2000; Liu et al., 1998). STAT4 and STAT1 activity is inhibited by PIASx and PIASy, respectively. They repress the STAT transcriptional activity by recruiting additional co-repressing factors (Arora et al., 2003; Liu et al., 2001). Recent reports describe PIAS proteins as SUMO E3 ligases that attach SUMO proteins to a number of target proteins. STAT1 was identified as a substrate for SUMO modification by PIASx. However, this particular modification of STAT1 was not essential for the PIAS mediated inhibition of its activity (Rogers et al., 2003; Schmidt and Muller, 2003).

4. SOCS proteins

At present the SOCS family of proteins counts eight different members, cytokine inducible SH2 protein (CIS) and SOCS1 through SOCS7. CIS is the founding member of the SOCS family and was cloned in 1995 by the group of Miyajima (Yoshimura et al., 1995). It was initially identified as an immediate early response gene induced in hematopoietic cells in response to Epo or IL-3 stimulation.

A few years later, the cloning of the second member of the SOCS family was reported simultaneously by three independent research groups, using three different approaches. One group identified SOCS1 while screening for inhibitors of cytokine signalling. A myeloid leukemic cell line was infected with a retroviral cDNA library and colonies failing to differentiate into macrophages upon IL-6 stimulation were selected and characterized (Starr et al., 1997). Another study used antibodies directed against the SH2 domain of STAT3 and identified the STAT-induced STAT-inhibitor-1 (SSI-1) (Naka et al., 1997). Finally, a yeast two-hybrid screening led to the detection of JAK binding protein (JAB) as an interaction partner of the catalytic domain of JAK2 (Endo 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). Although three parallel systems of nomenclature exist at the moment to address these genes and proteins, the SOCS nomenclature is most commonly used.

SOCS proteins are part of a classic negative feedback loop. SOCS proteins typically are not abundantly expressed in resting cells. They are induced upon stimulation by a broad range of cytokine and then interfere with cytokine signalling by blocking various components of the signalling pathway. The pattern of SOCS protein upregulation by a particular cytokine tends to vary according to the observed cell type or tissue (Krebs et al., 2001). The induced SOCS proteins attenuate signalling by various cytokine receptors. They can downmodulate the very signalling pathway that stimulated their production but can also inhibit signalling of other cytokines, mediating inhibitory cross-talk between cytokine signalling pathways. SOCS1, for example, is upregulated by IL-6 in CD4+ T-cells which then inhibits IFN-γ signalling and prevents T_H1 differentiation (Diehl et al., 2000). An overview of SOCS induction patterns and inhibition of cytokine signalling is given in table 3.
Recently, it was discovered that SOCS proteins can also be induced by other stimuli than cytokines. Toll-like receptors (TLR) are involved in recognition of pathogen-associated microbial components including LPS and can induce SOCS factors in for example macrophages (Heeg et al., 2003; Nakagawa et al., 2002).

STAT proteins are the predominant regulators responsible for the cytokine induced activation of SOCS gene expression. STAT binding sequences were identified in the promoter region of SOCS proteins and electrophoretic mobility shift assays confirmed STAT association to these motifs (Auernhammer et al., 1999; Emanuelli et al., 2000; Verdier et al., 1998).
The promoter of CIS carries four STAT5 binding sites all involved in Epo-dependent CIS induction (Matsumoto et al., 1997). A STAT1/STAT3 binding element in the promoter of SOCS3 interacting with both STAT1 and STAT3 is responsible for LIF-induced SOCS3 expression whereas IFN-γ-dependent SOCS3 upregulation relied on STAT1 homodimer binding (Auernhammer et al., 1999; Gatto et al., 2004). STAT5b also interacts with this STAT binding element previously identified as a STAT1/STAT3 binding site in the SOCS3 promoter and is involved in SOCS3 induction since mice lacking STAT5b show reduced GH induced SOCS3 expression (Davey et al., 1999; Emanuelli et al., 2000). Putative binding sites for STAT3 and STAT6 were detected in the promoter of SOCS1 and a dominant negative STAT3 mutant blocked LIF or IL-6 triggered induction of SOCS1 (Naka et al., 1997). In some cases, STAT-induced

SOCS expression is indirect. For example, STAT1 is indirectly involved in IFN-γ induced upregulation of SOCS1 since it induces expression of the interferon regulatory factor-1 (IRF-1) transcription factor which is responsible for stimulation of SOCS1 transcription (Saito et al., 2000).

The timing and intensity by which SOCS proteins are upregulated influence the kinetics of the particular cytokine activity (Krebs et al., 2001). It was demonstrated that while both IL-6 and IFNγ induce SOCS1 and SOCS3 expression in macrophages, IL-6 induces particularly SOCS3 in a very rapid manner while IFNγ upregulates mainly SOCS1 at a later time point. Accordingly, these cells were desensitized rapidly to IL-6 and later to IFNγ (Wormald et al., 2006). While most SOCS proteins are usually upregulated relatively fast after cytokine stimulation SOCS2 induction typically occurs later and is maintained longer. We propose that SOCS2 interferes with other SOCS proteins and this will be discussed in more detail in part III.

Table 3: SOCS cross-regulation, induction and inhibition patterns

SOCS proteins are part of a negative feedback loop. Their expression can be induced by a variety of cytokines and they inhibit signalling by several cytokine receptors. (adapted from Fujimoto et al., 2003)

SOCS	*Induced by*	*Inhibits signaltransduction of*
CIS	Leptin, Epo, GH, PRL, IL-2, IL-3, IL-6, IFN-α, IL-9	Leptin, Epo, GH, PRL, IL-2, IL-3
SOCS1	Epo, GH, PRL, insulin, G-CSF, GM-CSF, IL-2, IL-3, IL-6,LIF, IFN-α/β, IFN-γ	Leptin, Epo, GH, PRL, insulin, IL-2, IL-6, LIF, IFN-α/β, IFN-γ
SOCS2	GH, PRL, insulin, IL-6, LIF, IFN-α, IFN-γ, IL-9	Leptin, GH, PRL, IL-6, LIF, IFNγ
SOCS3	Leptin, Epo, GH, PRL, insulin, GM-CSF, IL-2, IL-6, LIF, IFN-α, IFN-γ, IL-9	Leptin, Epo, GH, PRL, insulin, IL-2, IL-6, LIF, IFN-α/β, IFN-γ, IL-9
SOCS4	EGF	
SOCS5	EGF, IL-6	EGF, IL-4, IL-6
SOCS6	insulin	insulin
SOCS7	GH, PRL, insulin, IL-6, IFNγ	Leptin, GH, PRL, insulin

Physiological functions of SOCS proteins

Mediation of the complex network of cytokine signalling involves SOCS proteins. They are indispensable in homeostasis of many biological functions. The role of SOCS proteins has been extensively studied both in *in vitro* experiments and *in vivo* in loss-of-function or transgenic mouse models. The main characteristics of SOCS deficient and transgenic mice are summarized in table 4.

Table 4: Phenotype of SOCS deficient mice and transgenic mice

SOCS	*Knock-out phenotype*	*Transgenic phenotype*
CIS	No defects	Defective in growth, immune response and mammary gland development
SOCS1	Neonatal lethality, severe defects in immune system	Defects in T-cell development
SOCS2	Gigantism	Gigantism
SOCS3	Embryonic lethality	Embryonic lethality
SOCS4		
SOCS5	No defects	Defective T_H2 responses
SOCS6	Mild growth retardation	Improved insulin and glucose tolerance
SOCS7	50% lethality attributed to hydrocephalus	

CIS

CIS is ubiquitously expressed. Northern blot analysis revealed strong expression levels in liver, kidney, lung, spleen, heart and testis (Starr et al., 1997).

Deficiency of CIS did not lead to any detectable defects in mice. CIS transgenic mice were smaller than wild-type mice and manifested defects in mammary gland development and immune responses (Matsumoto et al., 1999). A remarkable resemblance, observed between the phenotype of these CIS transgenic mice and that of STAT5 knockout mice suggests that CIS acts as a specific negative feedback regulator of STAT5 activity (Matsumoto et al., 1999; Teglund et al., 1998). Indeed, CIS is induced by several STAT5 mediated cytokine receptor signalling systems including Epo, IL-2, IL-3, GH and PRL and was also identified as an inhibitor of these

particular systems (Aman et al., 1999; Dif et al., 2001; Matsumoto et al., 1997; Pezet et al., 1999; Ram and Waxman, 1999; Verdier et al., 1998; Yoshimura et al., 1995).

SOCS1

High expression levels of SOCS1 mRNA are observed in murine thymus tissue. SOCS1 is also rapidly induced in mouse liver upon IL-6 stimulation (Starr et al., 1997). Hypermethylation within the promoter region of SOCS1 results in downregulation of SOCS1 expression which may be implicated in cancer development (To et al., 2004; Yoshikawa et al., 2001). SOCS1 induction was demonstrated to be induced by multiple cytokines, including IFNγ, GH, PRL, IL-2, IL-6 and Epo (Adams et al., 1998; Pezet et al., 1999; Sakamoto et al., 1998; Sporri et al., 2001; Starr et al., 1997; Sarna et al., 2003). Strong inhibition of GH-or PRL-dependent STAT5 activation and of EpoR signalling is mediated by SOCS1 (Adams et al., 1998; Dif et al., 2001; Hansen et al., 1999).

Three independent research groups generated mice lacking SOCS1 to investigate the *in vivo* function of SOCS1 (Marine et al., 1999b; Naka et al., 1998; Starr et al., 1998). SOCS1 deficient mice suffer from a complex disease characterized by deregulation of the immune system at multiple levels and die within three weeks after birth (Naka et al, 1998; Starr et al., 1998). They exhibit stunted growth and suffer from fatty degeneration and necrosis of the liver, severe lymphopenia, monocytic infiltration of major organs and peripheral T cell activation. Surprisingly, defects in these mice are primarily due to uncontrolled IFNγ signalling and consequent constitutive STAT1 activity. Administration of neutralizing anti-IFNγ antibodies largely prevents the onset of the complex disease associated with SOCS1 deficiency (Alexander et al., 1999). Moreover, SOCS1 knock-outs that also lack the IFN-γ gene were rescued from neonatal lethality, confirming the inhibitory function of SOCS1 in the STAT1 dependent IFNγ signalling pathway (Alexander et al., 1999). *In vitro* antiproliferative and antiviral activities of IFN were also inhibited by SOCS1 (Song et al., 1998).

SOCS2

Relatively weak expression of SOCS2 is demonstrated predominantly in murine liver, testis and lungs (Metcalf et al., 2000; Starr et al., 1997). SOCS2 expression is upregulated upon stimulation with IL-6, Epo, IL-3, PRL, GH, GM-CSF, G-CSF and IFNγ (Adams et al., 1998; Pezet et al., 1999; Starr et al., 1997). Signal transduction of GH,

PRL, IL-6 and LIF is repressed by SOCS2 (Minamoto et al., 1997; Nicholson et al., 1999; Pezet et al., 1999; Ram and Waxman, 1999).

Mice lacking SOCS2 exhibit gigantism associated with increases in bone and body length and augmented weight of organs and carcass. This phenotype is due to a prolonged GH dependent STAT5 activity (Greenhalgh et al., 2002a; Metcalf et al., 2000). Surprisingly, SOCS2 transgenic mice showed a similarly increased growth (Greenhalgh et al., 2002b). This dual effect of SOCS2 was also observed *in vitro*, were low SOCS2 doses moderately inhibit GH signalling while higher levels positively regulate signalling. It was hypothesized that SOCS2 blocks SOCS1-mediated inhibition of GH and PRL signalling and interferes with the downmodulation of PRL signalling by SOCS3 (Dif et al., 2001; Favre et al., 1999).

Unlike SOCS1 and 3 that are typically induced in a rapid and transient manner upon stimulation, SOCS2 expression usually is more prolonged (Pezet et al., 1999; Tollet-Egnell et al., 1999). Therefore, SOCS2 may be involved in restoring cellular sensitivity by overcoming the inhibitory effect of other SOCS proteins. This is discussed in more detail in part III.

SOCS3

SOCS3 expression is induced by a broad range of cytokines including Epo, G-CSF, GM-CSF, IL-3, IFNγ, leptin and PRL (Bjorbaek et al., 1998; Pezet et al., 1999; Starr et al., 1997). SOCS3 expression at lower levels is observed in lung and spleen and expression levels are induced in liver upon stimulation with leptin or IL-6 (Starr et al., 1997; Waelput et al., 2000). Signal transduction of several cytokines including GH, PRL, IL-2, IL-3, IL-6, G-CSF, IFNγ and Epo is suppressed considerably by SOCS3 (Cohney et al., 1999; Hansen et al., 1999; Hörtner et al., 2002b; Nicholson et al., 1999; Pezet et al., 1999; Sasaki et al., 2000; Song and Shuai 1998). SOCS3 was also identified as a potent inhibitor of LR signalling and is rapidly expressed in the hypothalamus upon leptin stimulation (Bjorbaek et al., 1998).

Both SOCS3-deficient and transgenic mice die in utero. Lack of SOCS3 results in a marked erythrocytosis and placental insufficiency while SOCS3 transgenic mice are completely deficient in foetal liver erythropoiesis (Marine et al., 1999a; Roberts et al., 2001). These observations suggest a critical function for SOCS3 in regulation of foetal liver haematopoiesis at the level of Epo signal transduction.

SOCS3 heterozygous or neural-cell specific deficient mice are viable. They show augmented leptin sensitivity in the hypothalamus associated with a remarkable attenuation of diet-induced obesity, suggesting a key role for SOCS3 in leptin resistance (Howard et al., 2004; Mori et al., 2004).

It was demonstrated that SOCS3 deficiency in macrophages and hepatocytes leads to increased activation of both STAT3 and STAT1 after stimulation with the proinflammatory cytokine IL-6, demonstrating its role in negative regulation of IL-6 signalling. This results in a STAT3-mediated IL-10 like immunosuppressive response and a STAT1-mediated IFN-like response, which changes the normal cellular IL-6 effect. Apparently, SOCS3 controls the IL-6 response in preventing an anti-inflammatory response (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003).

Other SOCS proteins

To date, studies addressing the physiological actions of the remaining four SOCS proteins are only beginning to emerge. Very little is known about SOCS4 so far. SOCS4 expression is upregulated upon EGF stimulation (Kario et al., 2005). Recently, it was reported that Mycobacterium tuberculosis infection in mice is associated with augmented levels of type I IFNs, SOCS4 and SOCS5 (Manca et al., 2005). SOCS5 is expressed in many tissues and especially in hematopoietic tissues (Magrangeas et al., 2000). T-cells from transgenic mice constitutively expressing SOCS5 demonstrate that SOCS5 inhibits IL-4 signalling and promotes T_H1 differentiation (Seki et al., 2002). Surprisingly however, SOCS5 knock-out mice exert a normal T_H1/T_H2 balance and show normal susceptibility to pathogen infection (Brender et al., 2004). SOCS5 expression is triggered by EGF and exerts a negative effect on its signal transduction (Kario et al., 2005; Nicholson et al., 2005).

SOCS6 and SOCS7 are expressed ubiquitously in murine tissues (Krebs et al., 2002). Mice lacking SOCS6 develop normally but weigh about 10% less than wild type mice. They exhibit no defects in hematopoiesis or glucose metabolism (Krebs et al., 2002). Contrary to SOCS6 knock out mice, SOCS6 transgenes displayed improvement in their glucose metabolism (Li et al., 2004). SOCS6 was shown to associate with the insulin receptor upon stimulation with insulin (Mooney et al., 2001). This association may occur indirectly since SOCS6 binding with IRS-4 and the p85 regulatory subunit of PI-3K was observed in response to insulin stimulation (Krebs et al., 2002).

Interaction of SOCS6 with IRS-4 and p85 was also demonstrated upon IGF-1 stimulus (Krebs et al., 2002). SOCS6 expression is induced by insulin and SCF (Bayle et al., 2004; Li et al., 2004). Interaction of SOCS6 with the KIT receptor is demonstrated upon SCF stimulation (Bayle et al., 2004). After insulin or SCF stimulation, an inhibitory effect is observed on ERK1/2 activation but not on the activation of Akt (Bayle et al., 2004; Li et al., 2004; Mooney et al., 2001). SOCS6 may even have a positive effect on Akt activation upon insulin stimulation which may involve PI-3K (Li et al., 2004). SOCS7 expression is induced by IL-6, PRL, GH, IFNγ and insulin (Banks et al., 2005; Dogusan et al., 2000; Van De Wiele et al., 2004). At birth, SOCS7 knock-out mice seem more or less normal. They have no defects in haematopoiesis or glucose and insulin concentration but are smaller than wild type mice (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, these mice develop hydrocephalus causing about 50% lethality (Krebs et al., 2004). Older mice also develop increased glucose tolerance and insulin sensitivity together with a mild hyperglycaemia and hyperinsulinemia (Banks et al., 2005). SOCS7 interacts with several components of the insulin pathway including IRS1, IRS4, p85 and the insulin receptor (Banks et al., 2005; Krebs et al., 2002). In addition, interaction of SOCS7 with PLCγ, cytoskeletal molecule vinexin and adaptor proteins Grb2 and Nck was demonstrated (Martens et al., 2004; Matuoka et al., 1997). Recently, SOCS7 was also implicated in negative regulation of signalling by leptin, GH and PRL (Martens et al., 2005).

Text Box: JAK-STAT signalling in human pathology

Cytokines stimulate biological responses in virtually all cell types throughout the body. They activate their specific receptor and signal predominantly via the JAK-STAT pathway that is highly regulated by SOCS proteins and other inhibitors. Obviously, dysregulations of the various components of this pathway are linked with a number of often inheritable diseases including immunological disorders and cancer. This is also reflected by the often severe phenotypes seen in knockout mice of the diverse signalling components. The JAK-STAT pathway is described in more detail throughout part I.

Despite the characteristic redundancy of cytokines many malfunctions on the level of a particular cytokine or its receptor do lead to severe abnormalities. Individuals with mutations in the genes encoding for leptin or its receptor suffer from extreme obesity and immunodeficiency (Clement et al., 1998; Ozata et al., 1999). The growth hormone insensitivity syndrome also termed Laron syndrome, a form of dwarfism, is caused by a variety of mutations or deletions in the GHR (Laron, 2004). Some forms of polycythemia or erythrocytosis, characterized by accumulation of red blood cells, are caused by mutations in the EpoR gene that result in a C-terminally truncated receptor (Arcasoy et al., 1997; Arcasoy et al., 2002; Gordeuk et al., 2005). Multiple EpoR splice variants were also identified in diverse human cancer cells (Arcasoy et al., 2003).

A number of diseases are associated with dysregulation of JAK or STAT activity. A variety of JAK3 loss-of-function mutations account for approximately 10% of inherited SCID characterized by profound T-cell lymphopenia and impaired B-cell activation. Since JAK3 is restricted to signalling through the γc receptor chain, these patients show no other defects than in immune cells/ immunity (O'Shea et al., 2004; Pesu et al., 2005). Mutations in other components of the γ-common pathway including γc, IL-7 and IL-7R, result in similar disease phenotype. Aberrant activity of JAK and STAT proteins often contributes to tumourigenesis. A somatic gain-of-function point mutation (V617F) constitutively activates JAK2 (Tefferi and Gilliland, 2005). Several chromosomal translocations fuse the JAK2 gene with other genes including TEL, BCR or PCM1 forming fusion oncogenes. These JAK2 fusion proteins are also strongly constitutively active (Valentino and Pierre, 2006). This constitutive activation of JAK2 both by gain-of-function point mutation and translocation leads to myeloproliferative disorders such as leukaemia. Recently, it was demonstrated that gain-of-function mutations in JAK3 are also involved in leukemogenesis (Walters et al., 2006).
Dysregulation of STAT activity, particularly STAT3 and STAT5, contributes to malignant cellular transformation. Various oncoproteins including Src can induce constitutive activation of STATs (Benekli et al., 2003; Bowman et al., 2000). Constitutively active STATs can exert their transforming activity through induction of antiapoptotic pathways and dysregulation of cell growth (Benekli et al., 2003; Valentino and Pierre, 2006). Constitutive STAT3 activity is associated with a number of human cancers including breast and prostate cancer, melanoma and leukaemia (Benekli et al., 2003).

Malfunctions in the negative control of the JAK-STAT pathway are also linked to human pathology. Transcriptional silencing by gene hypermethylation is demonstrated for SHP-1, SOCS1 and SOCS3 and is associated with cancer (Melzner and Möller, 2003; Valentino and Pierre, 2006). Several oncogenes are reported to repress SOCS-1 transcription. SOCS1 is thought to function as tumour suppressor gene involved in growth suppression and its silencing is linked with a variety of solid tumours and haematopoietic diseases (Valentino and Pierre, 2006; Yoshikawa et al., 2001).

SOCS protein structure and molecular mechanisms of SOCS protein action

Comparing amino acid sequences of the SOCS proteins revealed that CIS and SOCS2, SOCS1 and SOCS3, SOCS4 and SOCS5 and finally SOCS6 and SOCS7 form closely related pairs (Hilton et al., 1998). CIS and SOCS2 are the closest related with around 35% of amino acid identity while the rest of the SOCS pairs share approximately 25% of amino acid sequences. Remarkably, the genes for SOCS1 and SOCS3 have no introns in their coding sequence (Starr et al., 1997).

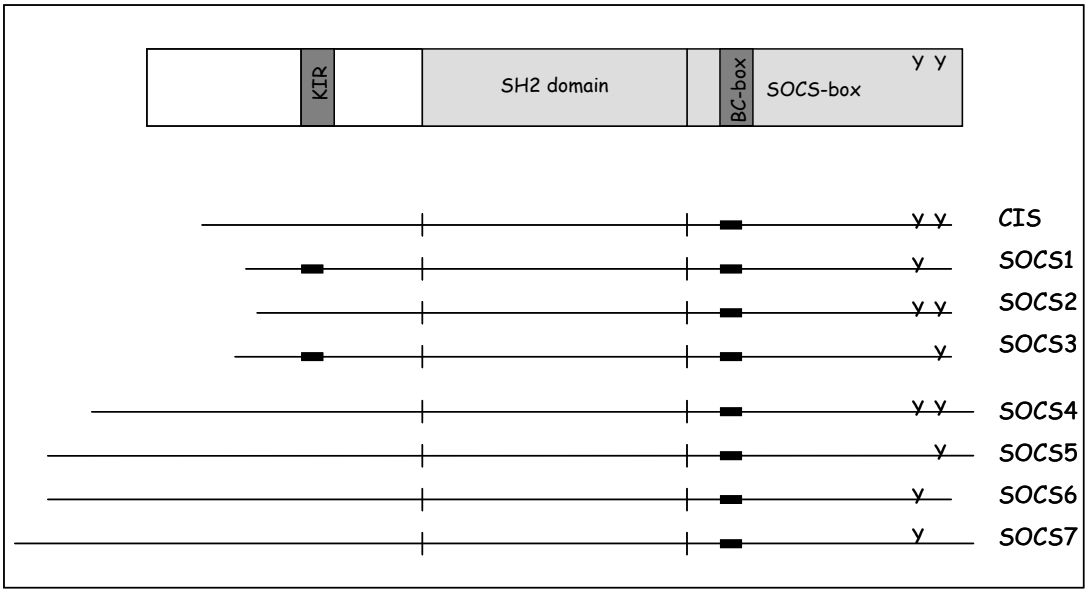

Figure 11: Schematic structure of the SOCS proteins
(adapted from Bullock et al., 2006)

The overall structure of SOCS proteins is well-preserved throughout evolution (figure 11). Additionally, SOCS homologues were identified in mammals, Drosophila melanogaster and Caenorhabditis elegans (Starr et al., 1997; Kile et al., 2002). SOCS proteins have a characteristic domain structure. They consist of a central SH2-domain, an N-terminal preSH2-domain and a C-terminal SOCS-box (Starr et al., 1997). The N-terminal domain varies in length and composition whereas the SH2 domain and SOCS-box are more conserved. The SOCS-box is thought to target

proteins for proteasomal degradation and the SH2 domain is responsible for the association with phosphotyrosine motifs imbedded in cytokine receptors or with other signalling proteins like JAK and IRS. In the N-terminal domain an ESS (extended SH2 subdomain) region was identified which is important for the binding capacity of the SH2 domain (Babon et al., 2006; Bullock et al., 2006; Nicholson et al., 1999; Sasaki et al., 1999; Yasukawa et al., 1999). This N-terminal domain also carries a KIR (kinase inhibitory region) in the case of SOCS1 and SOCS3.

SOCS family members can block cytokine signalling by several inhibitory mechanisms that are not mutually exclusive: blocking access of substrates by directly interacting with the receptor, inhibition of the intrinsic kinase activity of the JAKs and ubiquitination and proteasome targeting by the SOCS-box.

Hindering association of signalling molecules with the activated receptor

SOCS proteins can exert their negative effect by competing for phosphorylated tyrosine motifs in the receptor. They associate with these tyrosine motifs by means of their SH2 domain. The docking sites for signalling molecules including STATs become thereby inaccessible because of direct association or steric hindrance by the SOCS proteins, thereby blocking activation of downstream signalling effects. CIS is thought to inhibit STAT5 activation by association with Y401 of the human EpoR, which is one of the two recruitment sites for STAT5 (Verdier et al., 1998). CIS interaction was also observed with the membrane distal part of the GHR where the tyrosines involved in STAT5 activation are situated (Hansen et al., 1996; Ram et al., 1999). In addition, CIS is also engaged in negative regulation of PRLR signalling, involving steric hindrance (Endo et al., 2003). Little is known about the mechanism by which SOCS2 exerts its regulatory function. As mentioned before, it was demonstrated to function as an inhibitor in GH signalling at low concentrations although at higher concentrations it had a potentiating effect. SOCS2 can probably inhibit signalling in a similar way as CIS, involving interaction with the receptor. Association of SOCS2 with phosphotyrosine motifs of the GHR and the PRLR has been reported (Greenhalgh et al., 2002b; Pezet et al., 1999). Receptor association of CIS and SOCS2 is studied in more detail in part III.

Inhibition of JAK kinase activity

Only the SOCS1 and SOCS3 proteins are able to inhibit the activity of the JAK kinases by means of their N-terminal KIR domain (figure 12). This region was found functionally interchangeable between the two SOCS proteins suggesting a common inhibitory mechanism (Nicholson et al., 1999). The SH2 domain of SOCS1 is essential for direct interaction with the kinase domain of JAK2. It targets the phosphotyrosine at position Y1007 in the activation loop of JAK2 (Giordanetto et al., 2003; Yasukawa et al., 1999). Phosphorylation of this particular tyrosine is responsible for the activation of the kinase activity. The KIR domain of SOCS1 also contributes to the high-affinity binding with JAK2 but is also essential for the inhibitory function of the SOCS protein (Yasukawa et al., 1999). It associates with the catalytic groove of JAK2 and may obstruct the ATP binding pocket and hinder accessibility for substrates (Giordanetto et al., 2003; Yasukawa et al., 1999). Based on sequence similarity, the KIR domain somewhat resembles the activation loop of JAK2 (Yasukawa et al., 1999). It is suggested to act as a pseudosubstrate and mimic the activation loop that regulates access to the catalytic groove (Giordanetto et al., 2003; Yasukawa et al., 1999). Unlike SOCS1, SOCS3 showed only weak affinity for JAK2. It is thought to bind via its SH2 domain with phosphotyrosine motifs in the receptor in close proximity to the kinase to inhibit its activity through its KIR domain (Suzuki et al., 1998). Membrane proximal SOCS3 interaction was demonstrated for several cytokine receptors including LR, gp130, EpoR and GHR (Bjorbaek et al., 2000; Eyckerman et al., 2000; Hörtner et al., 2002a; Nicholson et al., 2000; Ram et al., 1999).

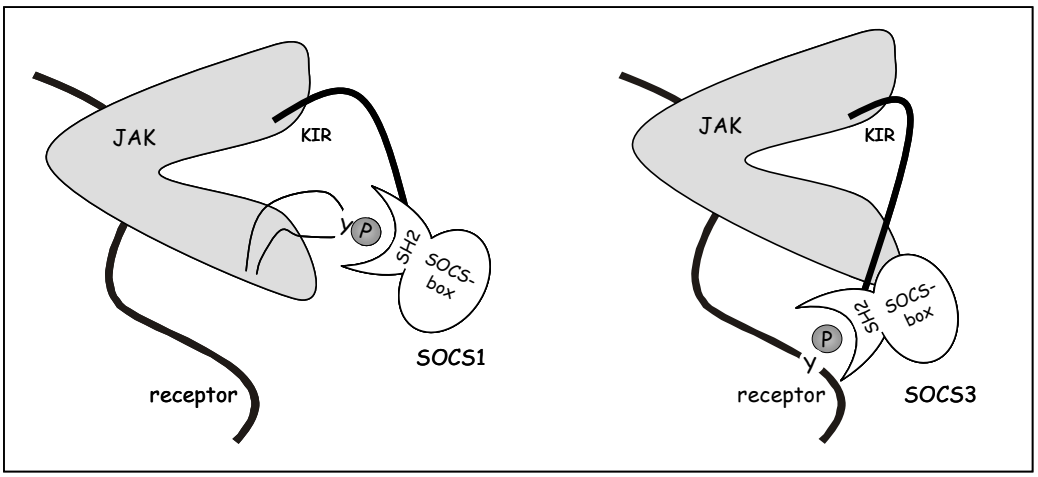

Figure 12: Model for molecular mechanism of SOCS1 and SOCS3 actions.

(adapted from Yoshimura et al., 2005)

The SOCS-box mediates proteasome targeting

The conserved SOCS-box domain of a variety of proteins has been shown to interact with elongins B and C (Kamura et al., 1998; Zhang et al., 1999). The VHL (Von Hippel-Lindau) protein was the first protein with a SOCS-box like domain that was reported to associate with elongin B and C (Duan et al., 1995). Elongin B and C association has also been reported for SOCS1, SOCS3 and SOCS6 and recently for SOCS2, SOCS5 and CIS (figure 13) (Bullock et al., 2006; Greenhalgh et al., 2005; Kamura et al., 1998; Kario et al., 2005; Krebs et al., 2002; Lavens et al., 2006; Zhang et al., 1999). The SOCS protein - elongin B/C complex forms an E3 ubiquitin ligase complex with other proteins like a Cul5 subunit and a ring finger protein Rbx (figure 14) (Kamura et al., 2004). Together with an E1 ubiquitin activating enzyme and the E2 carrier enzyme, this E3 ubiquitin ligase participates in the polyubiquitin tagging of associated substrate proteins and is responsible for selection of specific target proteins (Glickman et al., 2002). This way, SOCS proteins may inhibit signalling by marking associated signalling components for degradation (Kamura et al., 2001; Kamura et al., 2004). Consistent with this idea, proteasomal inhibitors induced sustained JAK-STAT signalling and block the inhibitory actions of SOCS proteins (Landsman and Waxman, 2005; Ram and waxman, 2000; Verdier et al., 1998). CIS interacts with the EpoR and GHR via phosphotyrosine motifs and is thought to target both activated receptors for degradation (Landsman and Waxman, 2005; Verdier et al., 1998). SOCS1 was demonstrated to mediate the proteasomal degradation of the constitutively active TEL-JAK2 as well as endogenous JAK2 in a SOCS-box dependent fashion (Frantsve et al., 2001; Kamizono et al., 2001; Ungureanu et al., 2002). Downstream signalling components like insulin receptor substrate (IRS)1, IRS2 or the Rac guanine nucleotide exchange factor Vav are also targeted for proteasomal degradation by SOCS proteins (De Sepulveda et al., 2000; Rui et al., 2002). Examination of IFNγ responses in knock-in mice expressing SOCS1 that lacks only the SOCS-box, confirmed that this domain is essential for its full signalling inhibition capacities (Zhang et al., 2001).

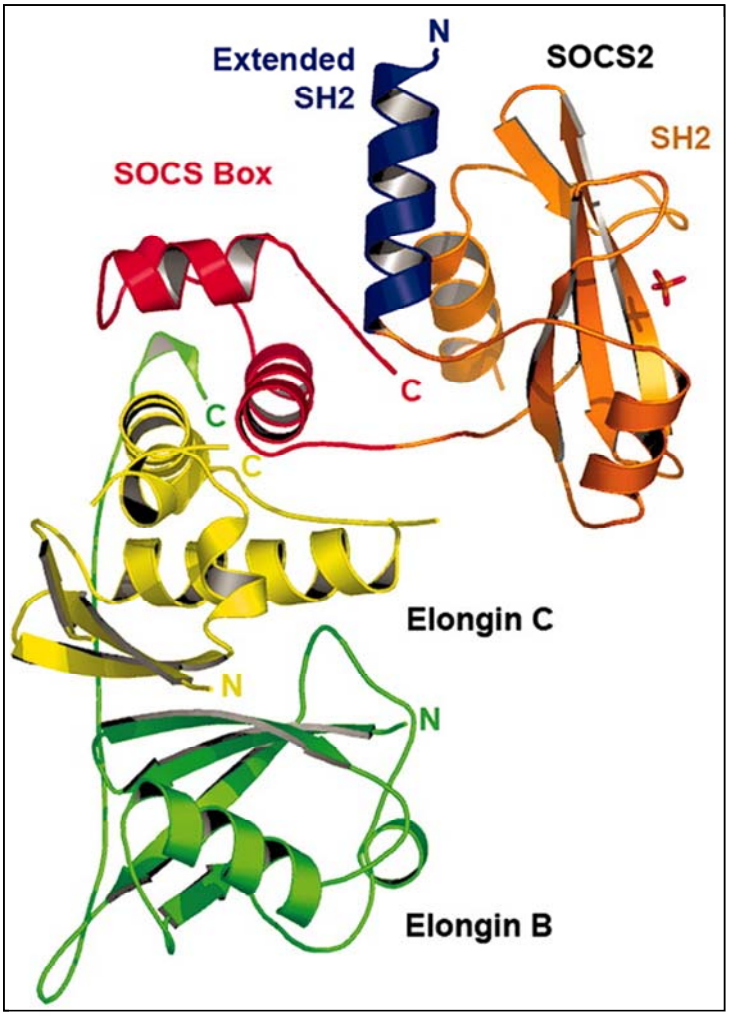

Figure 13: Three-dimensional structure of SOCS2 in complex with JAK2 and elongin B/C
(From Bullock et al., 2006)

The SOCS-box of the SOCS proteins clearly plays an important role in protein stability. Proteasome-mediated degradation is also proposed for SOCS proteins, including CIS and SOCS3 (Ram and Waxman, 2000; Zhang et al., 1999). A naturally occurring truncated form of the SOCS3 protein, lacking its main ubiquitination site, displays an increased stability because it is protected from proteasomal-mediated degradation (Sasaki et al., 2003). The effect of elongin binding on the stability of the SOCS proteins themselves is still a matter of debate. SOCS-box-mediated degradation of SOCS proteins has been suggested. Disruption of elongin B and C binding through phosphorylation of the SOCS-box positively affected SOCS1 stability (Chen et al., 2002). On the other hand however, some reports propose that the

interaction of the elongin B/C complex with the SOCS-box increases the stability of SOCS proteins. Proteasome-mediated degradation of SOCS3 was accelerated by disrupting the interaction of the SOCS-box with elongin C through tyrosine phosphorylation (Haan et al., 2003). An increased stability of SOCS1 mediated by elonginB/C complex interaction with the SOCS-box was also reported (Hanada et al., 2001; Kamura et al., 1998; Narazaki et al., 1998).

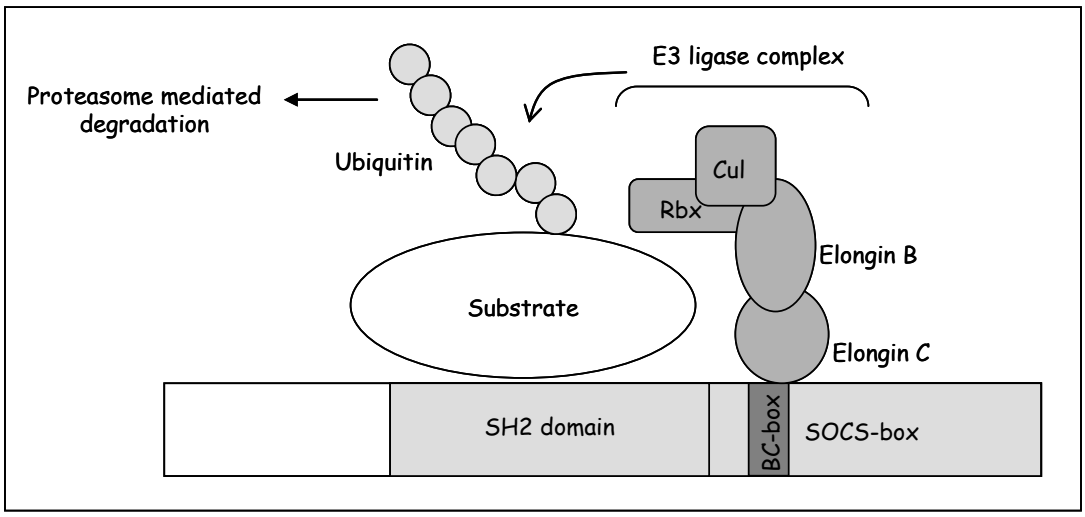

Figure 14: Model for proteasomal target degradation by SOCS proteins (Adapted from Elliott et al., 2004)

In some cases SOCS proteins have a positive regulatory role. SOCS6 is involved in increased AKT activation in response to insulin stimulation via interaction with the P85 monomer (Li et al., 2004). Positive SOCS effects are also observed in the MAPK pathway, as seen for CIS in activated T-cells (Li et al., 2000). Although SOCS3 negatively regulates JAK-STAT signalling, tyrosine phosphorylation in the SOCS-box of SOCS3 allows association of the Ras inhibitor $_{P}$120 RasGap and results in sustained extracellular-signal-regulated kinase (ERK) activation (Cacalano et al., 2001).
We demonstrate in this thesis that the SOCS-box can also be important for receptor association and for interference with other SOCS proteins and this will be discussed in more detail in part III.

5. Spred proteins

Sprouty (SPRY) proteins found in Drosophila inhibit signalling of several growth factors by suppressing the MAPK pathway. Four sprouty homologs were also found in mammalia (Kim and Bar-Sagi, 2004). Recently, related proteins with structural and functional similarities to SPRY were identified, named Sprouty-related EVH1 Domain-containing proteins (Spred). These proteins carry a sprouty-related cystein-rich (SPR) domain at their carboxyl terminus (Wakioka et al., 2001). So far 3 members of the Spred protein family were identified in mammals, Spred-1, Spred-2 and Spred-3. They are responsible for inhibition of the MAP kinase pathway involved in cytokine signalling. They suppress the phosphorylation and activation of Raf by interacting with Ras, but without reducing Ras activation (Wakioka et al., 2001). A C-terminally truncated Spred-1 isoform was identified and functions as a dominant negative variant, inhibiting the activity of its full size counterpart.

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Chapter 4: MAPPIT, a cytokine receptor based two-hybrid system

Protein interactions are involved in practically all processes in the cell. Protein complexes can form stable, cellular structures including the cytoskeleton and the proteasome complex. Protein interactions are also involved in the regulation or control of cellular processes. These interactions are often of a temporary nature and can depend on modification of one of the binding partners. Identification of the interaction partners of a particular protein can contribute considerably to identifying its functionality and to situating it in a specific context.

Protein interactions can be identified by two major kinds of approaches, biochemical or genetic methods (Xia et al., 2004). In a biochemical analysis proteins are co-purified and interaction partners are identified. Classic examples are peptide affinity chromatography and co-immunoprecipitation. In recent years, the introduction of sensitive and adequate protein identification by mass spectrometry led to the development of high-throughput biochemical techniques relying on tag-based affinity purification such as tandem affinity purification (TAP) (Puig et al., 2001). After cellular expression of a tagged bait protein and cell lysis, the complex is precipitated in one or more purification steps. Proteins are separated by protein gel electrophoresis and identified by mass spectrometry and subsequent database searching. For instance, this method was used to identify new components of the TNF-α signalling pathway (Bouwmeester et al., 2004).

These techniques allow detection of whole protein complexes. However, the inevitable lysis of the cells may cause disruption of weak interactions. Total disruption of the cell architecture can also result in illegitimate interactions between proteins that normally reside in separate compartments in the cell.

In genetic approaches both interacting proteins are adapted to functional molecules in a way that their specific interaction in intact cells leads to a detectable signal. The yeast two-hybrid technique based on reconstruction of a functional transcription factor was the first in vivo method. This system has been used extensively to characterize many protein interactions. In recent years, a high-throughput approach was applied to address protein interactome analysis in for example yeast and humans (Ito et al., 2000; Ito et al., 2001; Rual et al., 2005). This method suffers from a number of limitations due to the fact that proteins of higher organisms often need posttranslational modifications that are hard to reproduce in yeast and

interactions have to occur in the nucleus which eliminates membrane bound proteins or large proteins as candidates for examination. Use of mammalian cell systems can overcome some of the problems associated with the yeast two-hybrid technique (Eyckerman and Tavernier, 2002). The first mammalian two-hybrid systems were based on the Ras recruitment system and transcription factor complementation. Another mammalian genetic approach is the enzymatic complementation technique that is based on the reassembly and activation of two non-functional parts of a protein through interaction of the two attached proteins (Johnsson and Varshavsky, 1994). The fluorescence resonance energy transfer (FRET) method relies on the energy transfer between two different fluorophores to monitor the interaction or dissociation of the attached proteins. Light emission of the excited donor fluorophore results in excitation of the acceptor fluorophore only when both fluorophores are in close proximity (Wallrabe and Periasamy, 2005).

MAPPIT (mammalian protein-protein interaction trap) is a cytokine receptor based tool for detection of protein interactions in mammalian cells that was recently developed in our laboratory (Eyckerman et al., 2001). A detailed overview of cytokine receptor activation and signalling through the JAK-STAT pathway can be found in chapter 2. The MAPPIT two-hybrid method is based on LR signal transduction. Typically, a chimeric receptor consisting of the extracellular part of the EpoR and the intracellular part of the LR is deprived of its tyrosines to prevent STAT activation. A specific bait protein is attached C-terminally to this mutant chimeric receptor. MAPPIT prey constructs are composed of a prey protein coupled to a part of the cytoplasmatic tail of the gp130 chain carrying several STAT3 recruitment domains. Interaction of bait and prey protein will lead to functional complementation of STAT3 activity, which results in induction of a STAT3 responsive rat Pancreatitis Associated Protein I (rPAPI) promoter-luciferase reporter gene (Figure 15).

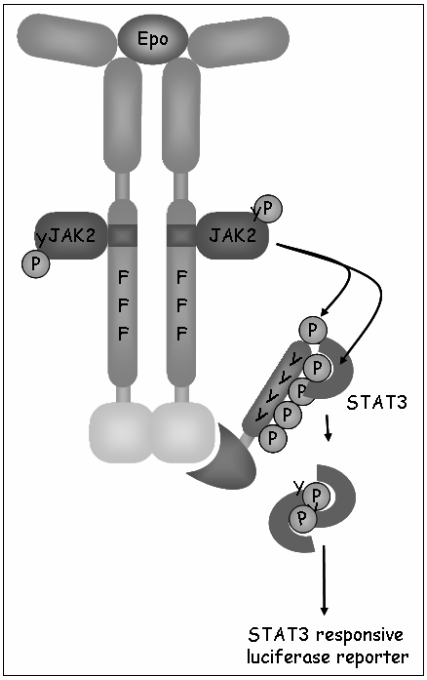

Figure 15: MAPPIT

In the MAPPIT system, a bait protein is coupled C-terminally to a mutant chimeric receptor containing the extracellular part of the EpoR and the transmembrane and intracellular part of the LR of which the STAT3 recruiting tyrosine at position Y1138 is mutated to phenylalanine. Tyrosine Y985 en Y1077 are also removed to prevent negative feedback and to maximize the intensity of the signal. MAPPIT prey proteins are linked to a part of the cytoplasmic tail of the gp130 receptor carrying multiple STAT3 recruitment sites. When bait and prey interact, phosphorylation of the prey chimeras 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 (Eyckerman et al., 2001).

The mammalian cell context of the technique offers substantial advantages. It provides a physiologically optimal context to examine protein interactions of higher order organisms, that often require specific posttranslational modifications of the proteins concerned. Moreover, intrinsic to this strategy, both modification-independent and JAK2-mediated tyrosine phosphorylation-dependent interactions can be detected. MAPPIT is therefore very suitable for studying protein interactions implicated in signalling pathways.

The MAPPIT technique keeps false positives to a minimum. It physically separates the interaction and detection zones in respectively the cytosol and the nucleus. This way bait and prey proteins can not interfere with the transcription of the reporter gene, since signalling read out is mediated by the endogenous STATs. In addition, the MAPPIT technique is an inducible system based on cytokine stimulation which allows elimination of ligand-independent, false positive interactions. The cytokine receptor-based setup of the method is suited for the identification of transient or, like mentioned earlier, modification-dependent interactions, thus reducing the amount of false negative interactions.

Various alternative versions of the MAPPIT technique were developed. Both the LR-MAPPIT and GGS-MAPPIT method were used in the context of this doctoral thesis (figure 16). The LR-MAPPIT system was developed to identify interaction partners of the LR. The LR itself, devoid of its STAT recruiting Y1138 tyrosine, is used as bait protein. The GGS-MAPPIT technique was created to avoid interfering interactions due to association with the LR. Therefore the intracellular part of the LR following the docking site of the JAK kinase is replaced by 60 GGS triplet repeats.

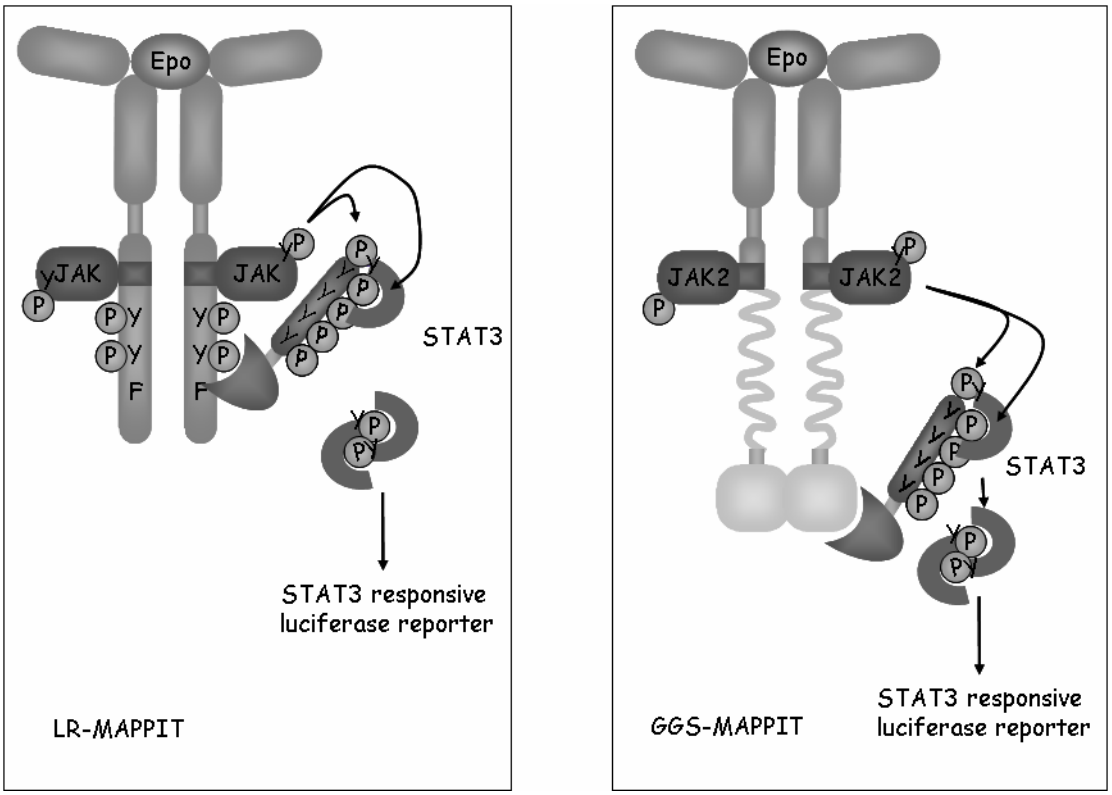

Figure 16: Alternative versions of MAPPIT: LR-MAPPIT and GGS-MAPPIT

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part II

Leptin: biological functions and signalling

Chapter 5: biological functions of leptin

1. Obesity, an expanding health problem in the twenty-first century

Despite increasing social pressure towards slimness, the number of people suffering from obesity is growing.

A simple formula that assesses obesity is the body mass index (BMI). It correlates body weight with height and is calculated by dividing the weight (in kilograms) by the square of the body length (in meters). An individual with a BMI of 30 or more is considered obese (table 5). The number clinically obese is estimated to exceed 300 million people worldwide. In the United States 60 % of the adult population is overweight (Friedman et al., 2004). This increasing prevalence of obesity forms a considerable health problem since it is associated with an augmented susceptibility for numerous diseases including type I diabetes, insulin resistance, hypertension, cardiovascular disease and some cancer types.

Obesity was long considered a psychological problem, a simple lack of will power. In recent years however, it has emerged from twin studies and experiments using mouse models that both genetic and environmental factors are involved. It is now clear that the tendency towards obesity is heritable, equivalent to that of height and more than many other conditions that are considered to have a genetic propensity including breast cancer or schizophrenia (Friedman, 2004).

Table 5: Body Mass Index

(classification according to the World Health Organisation)

BMI	*classification*
< 18.5	Underweight
18.5 – 24.9	Normal range
≥ 25	Overweight
25 – 29.9	Pre-obese
30 – 34.9	Obese class I
35 – 39.9	Obese class II
≥ 40	Obese Class III (morbid obesity)

2. Leptin and its receptor

Leptin is deduced from the Greek word leptos which means thin. It was identified by positional cloning as the protein product of the obese (*ob*) gene. This gene is truncated in the naturally occurring severely obese *ob/ob* mice (Zhang et al., 1994). These mice suffer from early-onset obesity, diabetes, decreased linear growth, infertility, and several other disorders. They weigh three times as much as wild type mice and their body fat content is increased five times (figure 17).

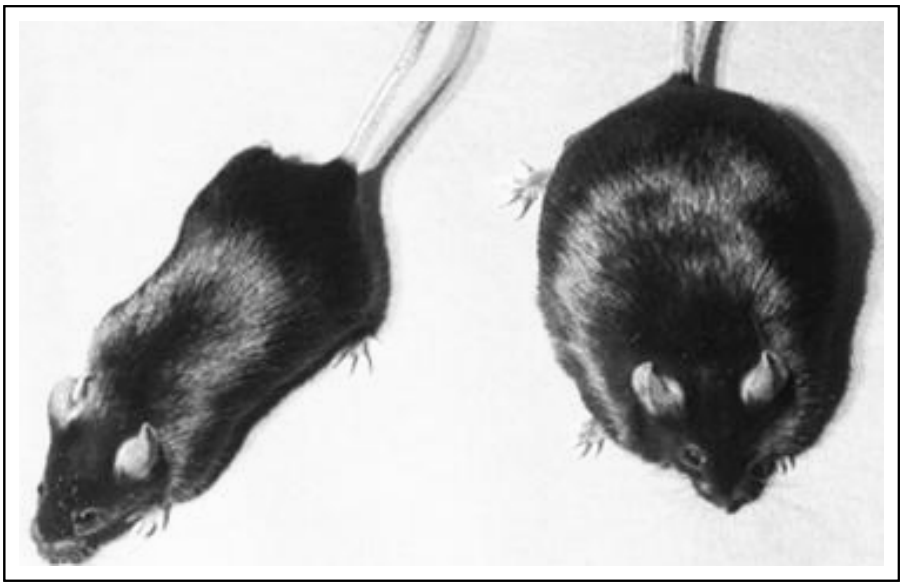

Figure 17: Phenotype of the *ob/ob* mouse (right).

Leptin is a secreted 167 amino acids long polypeptide. Its structure, which is presented in figure 18, was revealed by crystallography and resembles the structure of the long chain α-helical cytokines with a typical up-up-down-down folding (Zhang et al., 1997). The 16 kDa mature protein carries an intra-molecular disulphide bridge that is essential for its biological activity (Rock et al., 1996).

Leptin is secreted primarily by white adipose tissue and the plasma leptin levels correlate well with the amount of body fat (Considine et al., 1996; Maffei et al., 1995). Lower expression of leptin was also observed in other tissues such as the epithelium of the stomach and the placenta (Bado et al., 1998; Masuzaki et al., 1997).

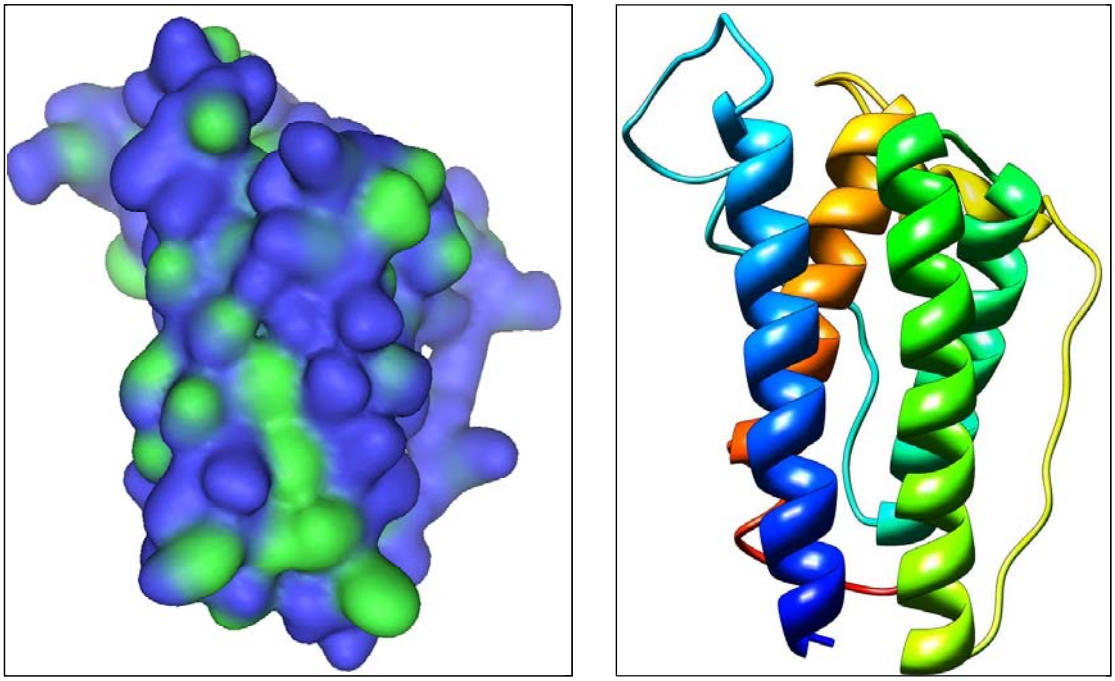

Figure 18: Structure of leptin, the molecular surface structure on the left hand side and the crystal structure on the right (adapted from Zhang et al., 1997).

Mice with the spontaneous *db/db* mutation display the same phenotype as *ob/ob* mice. Expressional cloning with a cDNA library revealed that the *db* (diabetes) gene codes for the receptor for the circulating leptin hormone (Tartaglia et al., 1995). The LR is part of the class I cytokine receptor family and has a very high sequence similarity with the G-CSFR and the gp130 receptor family, especially gp130, OSM and LIF receptors (Zabeau et al., 2003). At present, six isoforms of the LR have been identified, one long form (LRlo or LRb), one soluble variant (LRe) and four short forms (LRa or LRsh, LRc, LRd and LRf) and they are represented schematically in figure 19. The different LR variants are generated through alternative splicing but a soluble LR can also be generated by proteolytic ectodomain shedding of membrane-anchored LRs (Ge et al., 2002; Maamra et al., 2001). The different isoforms have an identical N-terminal, extracellular domain but differ in their C-terminal part. The extracellular domain consists of two CRH domains separated by an Ig domain and followed by two FNIII domains (figure 19). The membrane proximal CRH2 domain, the two FNIII-like domains and the Ig-like domain are required for leptin binding or receptor activation, the membrane distal CRH1 domain is strictly not essential but

does optimize signalling (Fong et al., 1998; Zabeau et al., 2004; Zabeau et al., 2005).

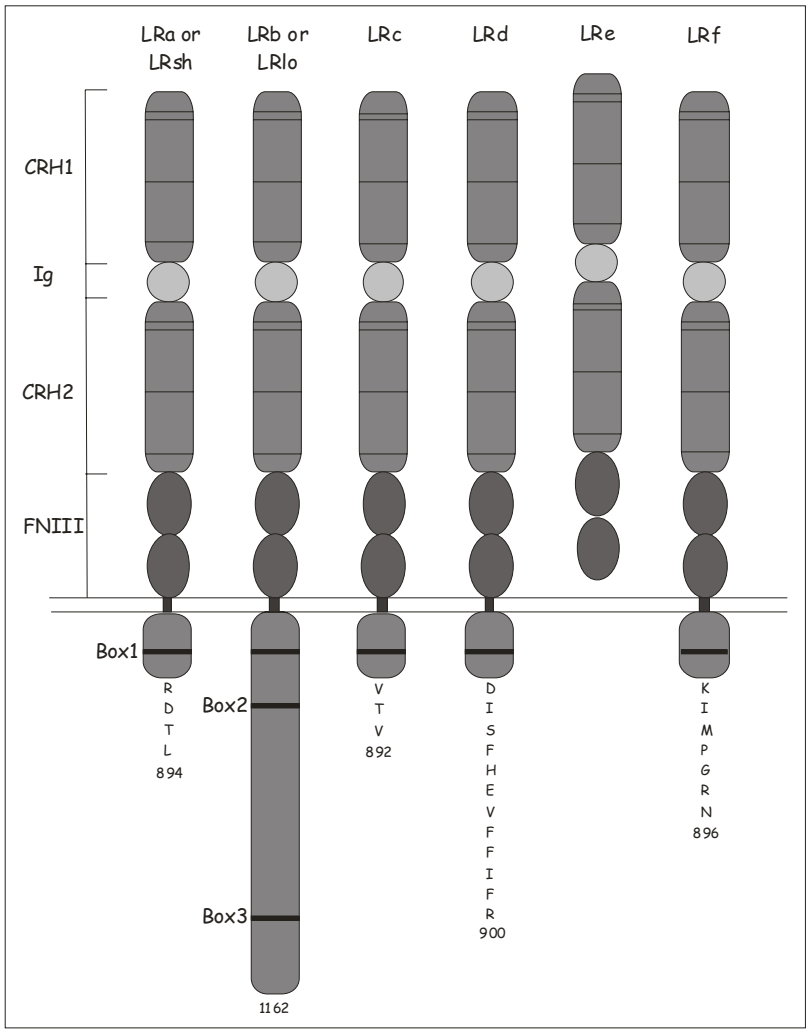

Figure 19: Structure of the LR isoforms.

The LRlo is expressed most abundantly in specific nuclei of the hypothalamus, but lower expression levels could be demonstrated in almost all tissues of the body (Dyer et al., 1997; Fei et al., 1997; Ghilardi et al., 1996; Mercer et al., 1996). It has a long cytoplasmatic domain and is predominantly responsible for signal transduction. In its intracellular tail it carries three conserved motifs, a membrane distal box 3 motif (YXXQ) that forms a docking site for STAT3 and two membrane proximal boxes, box1 and box2, that are involved in JAK2 association. The short isoforms of LR, especially the LRsh, are most abundantly expressed throughout the body (Fei et al., 1997; Ghilardi et al., 1996; Hoggard et al., 1997b). The LR short forms are thought

to contribute to the transport of leptin across the blood-brain barrier (Hileman et al., 2002). They are involved in leptin internalisation and subsequent degradation (Uotani et al., 1999). Furthermore, a role in leptin signal transduction was also suggested but this phenomenon remains questionable (Murakami et al., 1997). The soluble LR binds leptin with high affinity (Li et al., 1998). It is likely involved in determining the level of circulating leptin and in regulating the amount of free leptin available for signal transduction (Huang et al., 2001; Yang et al., 2004).

3. Energy homeostasis and leptin resistance

The body weight in adult individuals is remarkably constant despite the fluctuations in daily energy expenditure and food consumption. It is controlled very stringently by both short- and long-term homeostatic control systems. The short-term system mainly controls feeding via hunger and satiety signals. It involves glucose and amino acid concentrations in the plasma, body temperature, cholecystokinin and other hormones. The long-term system involves balancing feeding and energy expenditure and thereby eventually regulates energy homeostasis. Leptin is mainly a key player in the latter system but is also implicated in the short-term control where it is thought to play a role in satiation (Geary et al., 2004).

Since circulating leptin levels correlate accurately with the total body fat mass, leptin is considered to function as an adipostat that communicates the status of body energy reserves to the central nervous system (CNS) (Friedman and Halaas, 1998; Maffei et al., 1995). After leptin administration, mice increase their energy expenditure and decrease their food consumption (Halaas et al., 1995). Indeed, leptin forms an afferent signal of a negative feedback mechanism that keeps the body fat mass at a constant level. A drop in leptin levels due to a decrease in fat stores results in augmented food intake and lowered energy consumption while higher energy reserves correlate with elevated leptin levels, reduce food consumption and augment energy expenditure.

The importance of the brain, especially the hypothalamus, as a direct target in the weight reducing effects of leptin is demonstrated by neural-specific deletion of the LR which leads to obesity in mice (Cohen et al., 2001). To reach the CNS the adipocyte-derived leptin must thus pass the blood-brain-barrier (BBB). This presumably

involves a saturable system that might engage the short isoform of the leptin receptor which is highly expressed in the choroid plexus and cerebral microvessels (Banks et al., 2004; Bjorbaek et al., 1998; Devos et al., 1996; Tartaglia et al., 1995). In line with this, expression of the LRa in Madin-Darby canine kidney cells allowed directional transport of labelled leptin (Hileman et al., 2000). However, it was demonstrated that after brain perfusion leptin is transported at equal rates in wild type rats and Koletsky rats, which are deficient for all membrane-anchored LR isoforms. This might indicate that the LRsh is more a modulator of transport and the transporter itself remains to be identified (Banks et al., 2002).

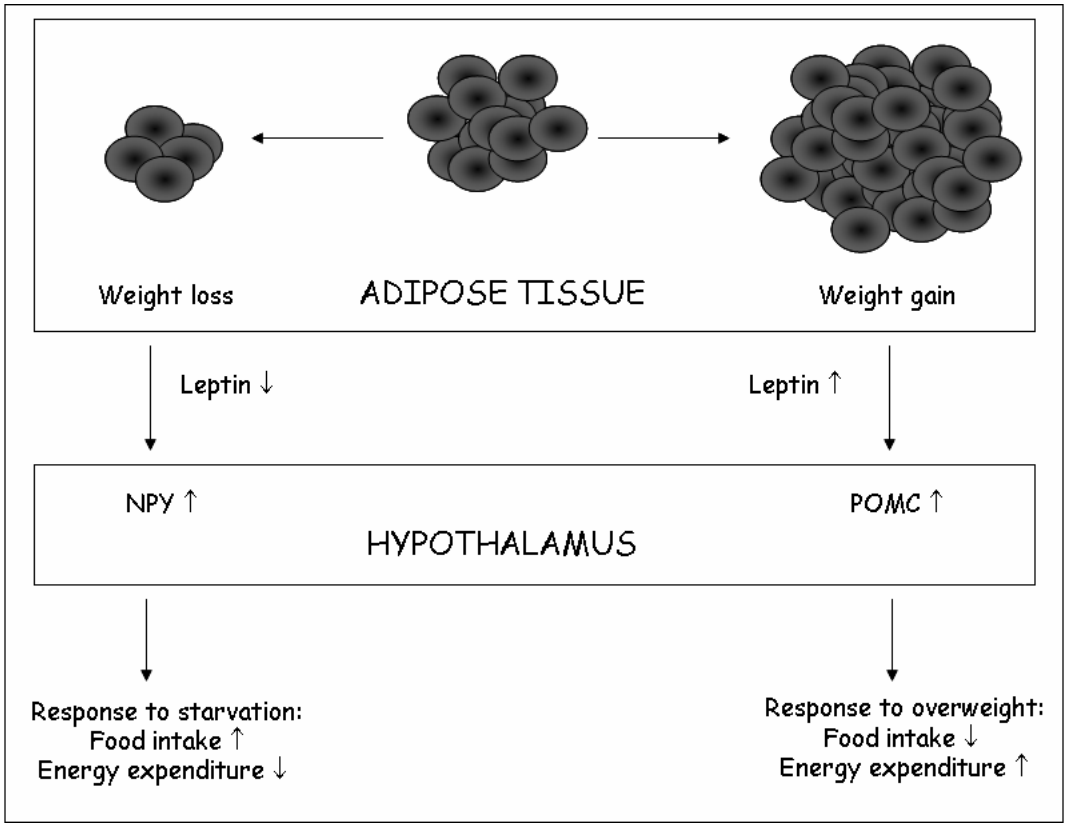

Figure 20: Leptin functions as an adipostat.

Leptin has an effect on two different populations of primary target neurons in the hypothalamus. The anorexigenic neurons express the satiety-related molecules cocaine-amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC), the precursor protein of the α-melanocyt-stimulating hormone (α-MSH). The orexigenic neurons are responsive to absence or low concentrations of leptin and express neuropeptide Y (NPY) and agouti-related protein (AgRP). The abundant

neurotransmitter NPY is a very potent orexigenic peptide that stimulates food consumption while AgRP antagonizes α-MSH action. Leptin induces expression of the anorexigenic molecules α-MSH and CART whilst it decreases NPY and AgRP levels (figure 20). 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).

Obviously deficiency in leptin leads to morbid obesity in humans but this phenomenon accounts for only a minority of obese individuals. In most cases, human obesity is associated with elevated leptin levels in the blood stream. Treating these patients with recombinant leptin has only minor effects on body weight loss (Heymsfield et al., 1999; Hukshorn et al., 2002). Apparently, they have developed a state of relative leptin resistance (Considine et al., 1996; Maffei et al., 1995). This leptin desensitisation is caused by defects in the leptin pathway and can be situated at three different levels (El-Haschimi et al., 2000; Münzberg and Myers, 2005):
First, the transport of leptin through the BBB can be impaired. A decreased transport of leptin across the BBB was demonstrated in rodents with diet-induced obesity (DIO) (Levin et al., 2004). Moreover, these DIO animals are resistant to peripheral leptin administration but lose weight after intracerebroventricular (ICV) injection of leptin, although central leptin signalling was not completely restored (El-Haschimi et al., 2000).
The latter observation suggests that leptin resistance can also be due to defects in hypothalamic leptin signalling. LR deficiency will evidently cause morbid obesity. In addition, aberrant signalling attenuation can cause central leptin insensitivity. SOCS3 and PTP1B are the two molecules that are most associated with modulation 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 knockout mice show hypersensitivity to leptin which protects them from high fat diet obesity (Elchebly et al., 1999; Howard et al., 2004; Mori et al., 2004). LR signal modulation is discussed in more detail in chapter 7.
A third mechanism that may underlie leptin resistance holds impairments in the downstream effects of leptin in the neuronal circuit. Defects may occur in the neuropeptides modulated by leptin such as NPY, AgRP, POMC or CART which were

addressed earlier. Exemplary is the obese phenotype observed for mice with null-mutations in the POMC encoding gene (Challis et al., 2004).

4. A broader role for leptin

Leptin function was originally thought to be restricted to the central regulation of body mass control. During recent years it has become more and more clear that it is a pleiotropic molecule and is involved in a diverse range of functions both in the CNS and in the periphery. This is reflected in the phenotype of *ob/ob* mice: in addition to an obesity syndrome, these mice have many abnormalities such as infertility and an impaired immune function (Coleman et al., 1978). These particular defects can all be corrected by administration of recombinant leptin (Campfield et al., 1995; Chehab et al., 1996; Halaas et al., 1995; Howard et al., 1999; Faggioni et al., 2000). Apparently, *ob/ob* mice are in a state of alleged starvation. Food collection and consumption are strongly prioritized, while the expense of energy on certain other biological functions – such as reproduction and the immune system- is minimized. In this way leptin provides a link between the energy status of the body and other vital physiological functions.

In recent years, several studies have linked increased circulating leptin levels with infection and inflammatory status, which suggested a role for leptin in the immune system. Leptin was recognized as a pro-inflammatory cytokine that can act as an early acute-phase reactant (Fantuzzi and Faggioni, 2000; Sarraf et al., 1997). It operates in both innate and adaptive immunity (La Cava and Matarese et al., 2004). In innate immunity, leptin promotes chemotaxis of neutrophils and their secretion of oxygen radicals (Caldefie-Chezet et al., 2003). It affects monocytes and macrophages by inducing phagocytosis, release of pro-inflammatory cytokines and expression of adhesion molecules (Fantuzzi and Faggioni, 2000; Mancuso et al., 2002; Zarkesh-Esfahani et al., 2001). Leptin also acts on the development and activation of natural killer (NK) cells (Tian et al., 2002). In adaptive immunity, leptin promotes the generation, maturation and survival of T cells by reducing apoptosis and it shifts memory T cells towards a T_H1 response (Howard et al., 1999; Lord et al., 1998). *In vivo* studies on mouse models revealed that leptin action is implicated in the pathogenesis of several autoimmune diseases including rheumatoid arthritis,

inflammatory bowel disease and multiple sclerosis (reviewed in Peelman et al., 2004).

It is well known that impaired reproductive function can be linked with both lack and excess of body fat. Leptin seems to be involved in pubertal development since administration accelerates the onset of puberty in female mice (Ahima et al., 1997; Chehab et al., 1997). It is also thought to play a role during pregnancy and lactation, although its exact functions in reproduction are still unclear (Brann et al., 2002). Moreover, a role for leptin was also suggested in foetal growth and development (Hoggard et al., 1997a; Masuzaki et al., 1997).

Since both leptin-deficient and LR-deficient mice show increased bone mass, an important role for leptin was expected in bone development. Leptin regulates bone formation both directly and indirectly via the CNS. It modulates activity of osteoblasts responsible for bone resorption and osteoclasts involved in bone formation (Ducy et al., 2000; Elefteriou et al., 2005; Gordeladze and Reseland, 2003; Martin et al., 2005; Thomas et al., 2005).

Finally, leptin is involved in cell proliferation and migration. It induces neovascularization and wound healing and may also influence the invasive capacity of cancer cells (Attoub et al., 2000; Murad et al., 2003; Schäfer et al., 2004; Sierra-Honigmann et al., 1998).

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Chapter 6: Leptin receptor signal transduction

THE INS AND OUTS OF LEPTIN RECEPTOR ACTIVATION

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Review published in FEBS Letters

FEBS 27257 FEBS Letters 546 (2003) 45–50

Minireview

The ins and outs of leptin receptor activation

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Received 5 March 2003; revised 28 March 2003; accepted 30 March 2003

First published online 1 May 2003

Edited by Richard Marais

Abstract The adipocyte-derived hormone leptin signals the status of body energy stores by activating its receptor in hypothalamic nuclei. In contrast to the initial expectations, leptin treatment of human obesity was largely unsuccessful. One explanation for this is the marked leptin resistance, which likely operates in part at the receptor level. The leptin receptor is a member of the class I cytokine receptor family, which uses the Janus kinase/signal transducer and activator of transcription pathway as a major signaling route. In this review, we focus on the molecular mechanisms underlying leptin receptor activation. Different modes of leptin-induced clustering of the ectodomains and the subsequent signaling events will be discussed.

Key words: Leptin; Leptin receptor; Receptor clustering; Signal transduction

1. Introduction

Leptin and its receptor are essential components in the complex genetic wiring diagram underlying energy homeostasis and body weight. Spontaneous mutations in leptin or in its receptor result in marked obesity in both mice [1,2] and man [3,4]. Leptin is mainly produced in white adipose tissue [2] although expression has also been demonstrated in the fundus of the stomach [5] and in skeletal muscle [6]. The long, signaling-competent isoform of the leptin receptor (LR) shows high expression peaks in the feeding centers of the hypothalamus [7], consistent with leptin being the afferent signal informing the central nervous system of the body fat status. This concept is further supported by the observation that leptin-deficient ($ob^{-/-}$) mice and men can be successfully treated with leptin [8,9]. Leptin was therefore initially considered a miracle drug for treatment of obesity. However, obese people often have elevated leptin levels [10] and leptin administration showed only very limited effects [11]. Recent data have indicated that this is likely due to desensitization for the leptin signal, a phenomenon now often referred to as leptin resistance. This may be situated at least at two distinct levels: saturable transport of leptin across the blood–brain barrier, and abnormalities at the level of LR activation and/or signal transduction [12]. Besides its role via the central nervous system, leptin also has direct effects on a series of peripheral tissues, implying a much more complex leptin axis than was originally anticipated [13].

To date, six splice variants of the LR have been identified. The long isoform or Ob-Rb (further referred to as LRlo) consists of 1162 amino acids and is the only LR isoform with clearly demonstrated signaling capability. It is highly expressed in hypothalamic centers although expression at functional levels has also been demonstrated in a number of other tissues including liver, lung, testis, etc. Neuronal-specific ablation of Ob-Rb results in obesity, clearly indicating that the weight-reducing properties of leptin are exerted centrally [14]. Four short isoforms (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf; Ob-Ra will be further referred to as LRsh) with shortened intracellular tails have been identified. High expression levels of Ob-Ra and Ob-Rc can be found in choroid plexus and brain microvessels [7], suggesting their role in blood–brain barrier transport. This idea is further supported by observations in mouse models for obesity [15] and by the use of an in vitro leptin transport assay [16]. A secreted isoform can be generated either by alternative splicing (Ob-Re) or by ectodomain shedding, and may be involved in modulating leptin activity [17].

2. Structure and evolutionary relationships of the LR extracellular domain

The LR was first cloned from a mouse choroid plexus cDNA library using an expression cloning strategy by Tartaglia and co-workers [7]. Based on sequence homology, this receptor belongs to the class I cytokine receptor family, which typically contains a so-called CRH (cytokine receptor homol-

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Abbreviations: CRH, cytokine receptor homology; FN III, fibronectin type III; G-CSF, granulocyte colony-stimulating factor; gp130, glycoprotein 130; Grb-2, growth receptor-bound-2; Ig, immunoglobulin; IL, interleukin; IRS, insulin receptor substrate; JAK, Janus kinase; LIF, leukemia inhibitory factor; LR, leptin receptor; MAPK, mitogen-activated protein kinase; OSM, oncostatin M; PI3K, phosphatidylinositol 3-kinase; PTP1B, phosphotyrosine phosphatase 1B; SHP-2, SH2-containing phosphatase-2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00440-X

ogy) domain in its extracellular domain. This structure consists of two barrel-like domains, each approximately 100 amino acids in length, which resemble the fibronectin type III (FN III) fold. Two conserved disulfide bridges are found in the N-terminal domain, while a WSXWS motif is characteristic for the C-terminal part. The LR shares highest sequence similarity with the granulocyte colony-stimulating factor (G-CSF) receptor and the glycoprotein 130 (gp130) family receptors, including gp130, the leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors. Moreover, structural superposition shows that also leptin is structurally most similar to G-CSF and cytokines of the gp130 family, such as interleukin-6 (IL-6).

Fig. 1A shows a consensus phylogenetic tree, based upon multiple sequence alignment of the membrane-proximal CRHs. The tree shown in Fig. 1 was calculated using the neighbor-joining distance method [18] with the Seqboot, Protdist and Neighbor programs of the PHYLIP package [19]. In this tree, the LR is most closely related to the OSM and LIF receptors, although the bootstrap support value for this evolutionary relationship is very low (40%). A parsimony tree, calculated using PAUP [20], shows an identical image, and supports the close evolutionary relationship between the LR and the LIF and OSM receptors (bootstrap support value 39%). A parsimony tree, calculated using the Seqboot and Protpars programs of the PHYLIP package [19] however, suggests that the LR would be more closely related to the G-CSF receptor and gp130 (bootstrap support value = 25%).

This relationship is also clearly reflected in the overall architecture of the ectodomains of these receptors (Fig. 1B): besides a canonical CRH domain, the receptors for leptin, G-CSF, LIF, OSM and gp130 all contain an immunoglobulin-like (Ig-like) domain (Fig. 1B). The LR and LIF receptors have an additional N-terminal CRH module, the OSM receptor has a truncated N-terminal CRH module. While gp130 and the G-CSF, LIF and OSM receptors each contain three membrane-proximal FN III domains, the LR stands out, having only two.

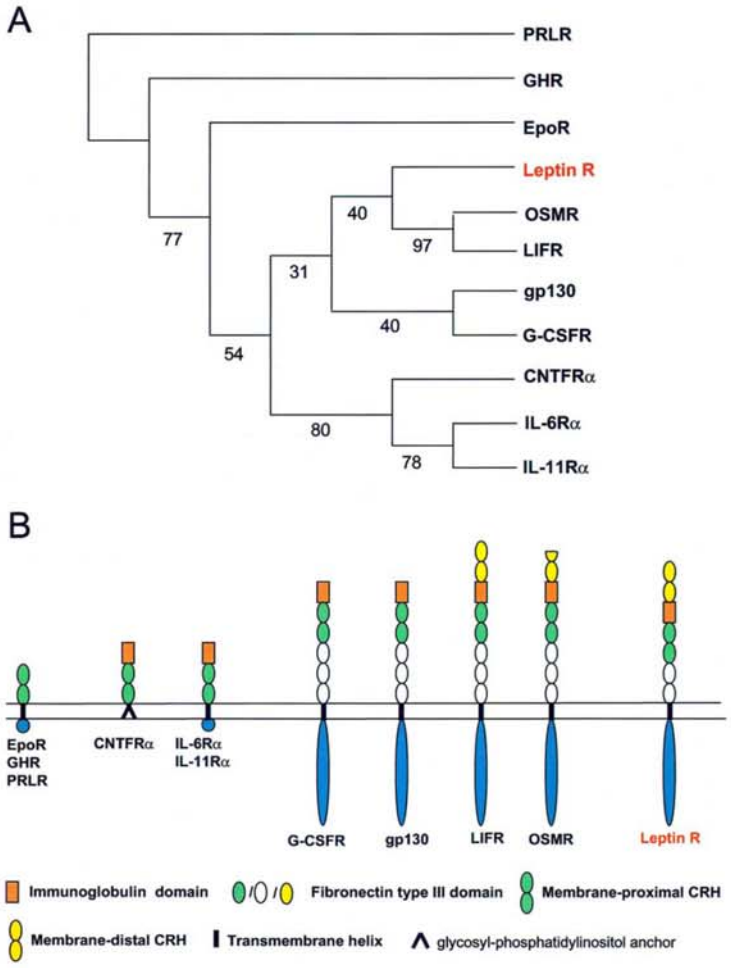

Fig. 1. A: Protdist phylogenetic tree of leptin and other long-chain cytokine receptors, based on an alignment of the membrane-proximal CRH. Numbers under the branches indicate the percent bootstrap support values out of 1000 bootstraps. PRLR: prolactin receptor; GHR: growth hormone receptor; EpoR: erythropoietin receptor; OSMR: oncostatin M receptor; LIFR: leukemia inhibitory factor receptor; G-CSFR: granulocyte colony-stimulating factor receptor; CNTFRα: ciliary neurotrophic factor receptor; IL-6Rα: interleukin 6α receptor; IL-11Rα: interleukin 11α receptor. B: Schematic presentation of the overall structure of long-chain cytokine receptors. The different domains are represented by colored squares or ovals, as explained below the figure.

3. Models for leptin/LR complex formation

Fong and co-workers generated a panel of LR deletion and substitution mutants, and showed that the membrane-proximal CRH domain is necessary and sufficient for leptin binding. These authors further provide evidence that the two FN III domains have no affinity for the ligand, but nevertheless are essential for receptor activation [21]. Recently, we could define a critical role for the Ig-like domain in receptor activation. Receptors lacking this domain are properly expressed on the cell surface, and bind leptin comparable to the wild type receptor, but are unable to activate the associated Janus kinases (JAKs) and fail to generate a signal transducer and activator of transcription (STAT)-3-dependent signal (see below; Zabeau et al., submitted).

Like all other class I cytokines, leptin adopts a four helical bundle structure. These cytokines usually interact with their receptor with two binding sites located on the helical faces of helices D (site I) and A and C (site II). A unique feature of G-CSF and of the gp130 family of cytokines is the presence of an additional binding site III, at the N-terminus of helix D, in one tip of the four helix bundle [22]. The function of this binding site III and of the Ig-like domain in the gp130 receptor systems was recently clarified by the crystal structure of the membrane-proximal CRH and Ig domain of gp130 in a 2:2 complex with Kaposi sarcoma herpesvirus IL-6 (vIL-6) [23]. This complex contains two copies of the gp130 fragment and of vIL-6. Each vIL-6 molecule interacts with two gp130 molecules by two interactions: binding site II in vIL-6 interacts with the CRH of one gp130 molecule, while binding site III in vIL-6 interacts with the Ig-like domain of a second gp130 molecule. This 2:2 tetrameric type of complex formation is very likely also a good model for the G-CSF:G-CSFR complex (Fig. 2A) [24]. Human IL-6 binds to its receptor in a 2:2:2 hexameric complex consisting of IL-6, IL-6Rα and gp130. In a generally accepted model, two human IL-6 molecules first bind to the CRH domains of dimeric IL-6Rα with their binding site I, and then bind to two gp130 receptor molecules with binding sites II and III [23,25] (Fig. 2B). It is tempting to speculate that similar clustering mechanisms also take place in the leptin/LR complex (Fig. 2C,D).

In gp130, the G-CSF receptor and the LIF receptor, the Ig-like domain interacts with binding site III of their cognate cytokine ligands. It is therefore very likely that a similar binding site III in leptin will interact with the Ig-like domain of the LR, which would help explain the deleterious effect of removal of this domain in the LR (Zabeau et al., submitted). Comparison with the IL-6 and G-CSF receptor systems suggests that leptin will bind its receptor either by its site I in the helical face of helix D, or by its site II in the helical faces of helices A and C. One of these sites probably interacts with the membrane-proximal CRH, as this constitutes the major leptin binding site [21]. Interactions between a binding site II in leptin and the membrane-proximal CRH were also suggested by molecular modeling of the leptin/LR complex [26,27].

Like the OSM and LIF receptors, LR contains an additional N-terminal CRH. In the LIF receptor, deletion of this CRH leads to a constitutively active receptor [28]. This is reminiscent of the constitutive activation seen in the fatty Zucker rat LR, which carries a mutation in this domain [29]. In the LIF receptor, this N-terminal CRH is able to interact with the ciliary neurotrophic factor receptor [30], suggesting a possible role for receptor/receptor interaction for the N-terminal CRH in the LR.

The membrane-proximal FN III domains of gp130 and of the LIF receptor interact with each other [31]. Although Devos et al. [32] showed that the membrane-proximal FN III domains are not necessary for dimerization of a soluble recombinant extracellular LR domain, similar interactions in the membrane-anchored LR cannot be excluded.

An important, unresolved question in LR biology is how LRlo can signal in the presence of excess LRsh. In many cell types, mRNA for the latter may account for up to 95% of all LR transcripts [33]. One possible explanation for this relative signaling insensitivity of LRlo to the effect of co-expression of dominant negative LR isoforms may be the formation of higher order clusters [29]. Based on the behavior of signal-

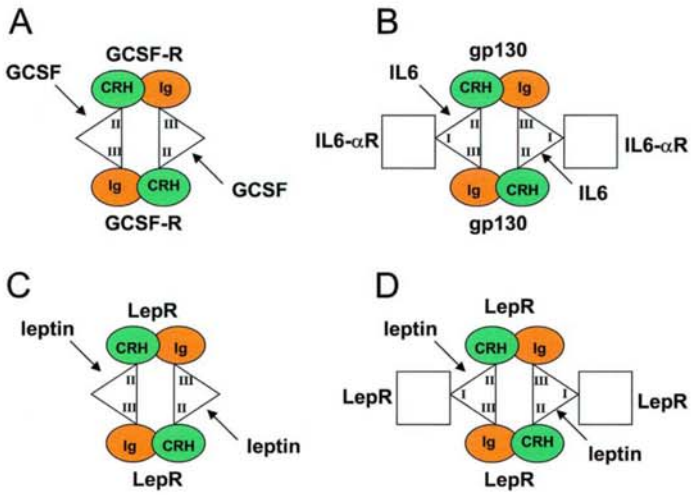

Fig. 2. Schematic representation of the complexes between cytokines and the extracellular domains of their receptors. A: G-CSF/G-CSF receptor 2:2 complex. B: IL-6/gp130/IL-6α receptor 2:2:2 complex. C: Model for a 2:2 leptin/LR complex. D: Model for a 2:4 leptin/LR complex.

ing-deficient LR mutants, we recently provided additional biochemical data to support this hypothesis (Zabeau et al., submitted). Like all other class I cytokine receptors, the LR lacks any intrinsic kinase activity, and uses cytoplasmic-associated kinases of the JAK family. Leptin binding results in formation of a receptor complex leading to cross-phosphorylation and activation of the JAKs. These activated JAKs then rapidly phosphorylate tyrosine residues in the cytosolic domain of the receptor. Such phosphorylated residues provide binding sites for signaling molecules including members of the STAT family. STATs themselves are also subject to JAK-mediated phosphorylation, inducing their homo- or heterodimerization, their release from the receptor complex, and subsequent translocation to the nucleus, where they can modulate transcription of specific target genes (for more details, see below). Two LR mutants, one deficient in the activation of JAK kinases (LR Δbox 1), the other unable to recruit STAT3 molecules (LR-F3), are only able to signal when they are co-expressed. Based on the requirements for JAK/STAT signaling, and on the lack of signaling complementation with similar receptor constructs, but containing the extracellular domain of the homodimeric erythropoietin receptor, this observation implies that more than two receptors must be clustered by leptin. When LRlo and LRsh forms are co-expressed, such higher order clustering can be expected to generate relatively more signaling-competent receptor complexes, when compared to simple homodimeric clustering. It is of note that the additional N-terminal CRH (or even the FN III domains) may contribute to even more complex patterns of receptor clustering.

An alternative explanation was suggested by White and coworkers [34]. These authors showed ligand-independent homo-oligomerization of LRlo and LRsh. Hetero-oligomerization between the two isoforms was only observed in the presence of ligand. Therefore, differential sorting, perhaps based on the ability to bind JAKs, of LRsh and LRlo may permit efficient leptin signaling by the latter form. Differential sorting of the two isoforms is also suggested by the observation that LRsh could be involved in active transcytosis of leptin [16].

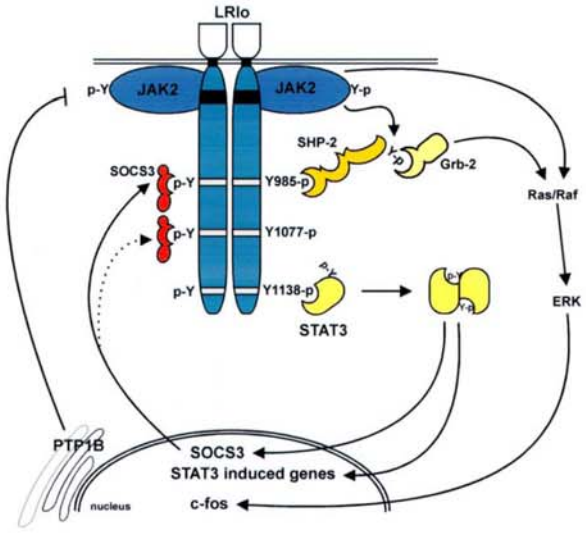

Fig. 3. LR signaling. The LR carries three conserved tyrosines in its cytoplasmic domain. JAK2, which associates membrane-proximally with the receptor, becomes activated upon ligand binding and phosphorylates these tyrosine residues. The membrane-distal Y1138 functions as a docking site for STAT3 which itself is a substrate of JAK2. Upon subsequent dimerization, it translocates to the nucleus and induces expression of SOCS3 and other genes. The membrane-proximal Y985 and Y1077 are involved in regulation and attenuation of the leptin signal. SOCS3 is taking part in a feedback loop inhibiting leptin signaling by binding to both tyrosines although binding affinity for Y1077 is much weaker. SHP2 is recruited to the Y985 position and activates the MAPK pathway through the adapter protein Grb-2, ultimately inducing c-*fos* expression. PTP1B is localized on the surface of the endoplasmic reticulum, and is also involved in negative regulation of LR signaling through dephosphorylation of JAK2 after internalization of the LRlo complex.

4. Activation of the JAK/STAT signaling pathway

A general overview of cellular events following LR activation is shown in Fig. 3. Leptin signaling occurs typically through the JAK/STAT pathway (see above). After ligand-induced clustering, the LRlo predominantly activates JAK2 [35], although JAK1 activation has also been demonstrated in some settings [36]. JAKs associate constitutively with a conserved box 1 motif, which is characterized by two essentially invariant prolines, in the class I cytokine receptors. Some receptors also contain an additional sequence, box 2, which is required for (maximal) JAK activation. In the murine LRlo, a box 1 motif (intracellular amino acids 6–17) critical for JAK2 activation and a putative box 2 motif (intracellular amino acids 49–60) have been identified. The latter may be required for maximal activation [35,37].

The LRlo has three conserved tyrosines in its cytoplasmic domain, which correspond in the murine receptor to positions Y985, Y1077 and Y1138. Y1138 is situated in a typical STAT3 recruitment or YxxQ motif, similar to motifs found in the gp130 family of receptors. After phosphorylation of this site, STAT3 is recruited via its SH2 (Src homology) domain. Activation, homo-dimerization and nuclear translocation of STAT3 will then lead to specific gene induction. The critical role of this site is underscored by the dramatic obese phenotype observed in knock-in mice containing a Y1138S mutation in LRlo [38]. It is unclear whether STAT3 is the only STAT factor that is activated upon stimulation. Vaisse and colleagues could demonstrate the activation of STAT3, but not of other STAT factors in the hypothalamus of leptin-treated *ob* mice [39]. In cell lines however, STAT1 and STAT5B activation was also shown [40]. The fact that LRlo Y1138S knock-in mice clearly have defects in body weight regulation but not in fertility, as opposed to mice lacking functional LRs, suggests alternative pathways, possibly via other STAT factors [38].

5. Role of phosphatases and of SOCS proteins

Mutation of the Y985 site in the receptor leads to enhanced signaling after leptin stimulation [36,41,42]. The Y985 site was identified as a recruitment site for the receptor-associated SH2-containing phosphatase-2 (SHP-2) [36,41]. It remains unclear whether negative effects are exerted via the phosphatase activity of SHP-2, or via the suppressor of cytokine signaling (SOCS)-3 protein, as it was later shown that this strong inhibitor of cytokine signaling also binds to the Y985 site [43,44]. SHP-2-dependent dephosphorylation of JAK2, how-

ever, also suggests an inhibiting function for SHP-2 in regulation of LRlo signaling [45]. SOCS proteins act in a typical negative feedback loop: they are rapidly induced after cytokine stimulation, and directly inhibit the receptor via various mechanisms, including receptor targeting to the proteasome. Both SOCS1 and SOCS3 can inhibit leptin signaling, and the observation that SOCS3 expression levels are elevated in the lethal yellow (A^y/a) obese mouse strain makes it a potential mediator of leptin resistance in vivo [46]. SOCS3 gene transcription is very rapidly induced in vitro and in vivo after leptin treatment [46,47], and even serves as a marker to map leptin-responsive neurons in the hypothalamus [48].

The function of the highly conserved Y1077 site is still unclear, mainly because phosphorylation of this site remains to be demonstrated. Remarkably, the Y985 and Y1077 sites are highly similar, suggesting binding of common signaling molecules. The Y1077 site shows weak interaction with SOCS3 and can therefore have an additive effect in signal termination [44].

SHP-2 is proposed as a positive regulator of leptin signaling through mitogen-activated protein kinase (MAPK) activation. Docking to Y985 is followed by recruitment of the adapter protein growth receptor bound 2 (Grb-2) and activation of the Ras/Raf pathway. A secondary pathway for leptin-induced MAPK signaling, directly via JAK2, probably requires the phosphatase activity of SHP-2. The MAPK pathway is responsible for leptin-induced c-*fos* activation [49]. It is of note that in LRlo signal transduction SOCS3 may also function as an adapter protein to other pathways [50].

Mice lacking PTP1B (phosphotyrosine phosphatase 1B) are hypersensitive to insulin and leptin and exhibit resistance to high fat diet obesity [51]. PTP1B recognizes a specific consensus substrate motif, (E/D)-$_p$Y-$_p$Y-(R/K), which was identified in JAK2 and the kinase activation loop of the insulin receptor [52,53]. Over-expression of PTP1B resulted in hypophosphorylation of endogenous JAK2 and blocked the leptin-induced transcription of endogenous SOCS3 and c-*fos* in a hypothalamic cell line. PTP1B appears to be a negative mediator of both the JAK/STAT and MAPK pathways in LR signaling and may be implicated in leptin resistance [54]. PTP1B is localized exclusively on the endoplasmic reticulum [55]. How PTP1B interacts with its substrates is not yet clear, although prior internalization of the receptor complex is suggested, especially since JAK2 has been detected at the endoplasmic reticulum [56].

6. Role of PI3 kinase

A strong correlation is assumed between the leptin and insulin signaling pathways since leptin and insulin resistance occur coincidentally in the majority of obese humans. Cross-talk between these pathways can be readily observed in various cell lines and in vivo. Phosphorylation of the insulin receptor substrates 1 and 2 (IRS1 and 2) as well as their interaction with Grb-2 and phosphatidylinositol 3-kinase (PI3K) show clear modulation by leptin in various hepatocytic cell lines [13,57]. Kim and colleagues performed a detailed in vivo study in rats showing STAT3 activation in insulin-responsive tissues after intraperitoneal injection of leptin. They also observed modulation of the PI3K and Grb-2 interaction with both IRS1 and IRS2 [58]. The apparent discrepancies described by various authors suggest that leptin and insulin signaling pathways interact in different ways depending on tissue type and cell line.

PI3K is also activated via IRS2 in the hypothalamus of rats, and appears to be crucial for the weight-reducing properties of leptin. Impaired PI3K signaling in peripheral tissues of obese individuals may also contribute to obesity-induced insulin resistance. A similar mechanism may explain the desensitization of leptin signaling in the hypothalamus, ultimately resulting in leptin resistance and obesity [59].

7. Concluding remarks

It is generally accepted that leptin plays a central role in regulating body weight. However, treatment of obesity using recombinant leptin seems to be only effective in individuals with a rare homozygous mutation in the gene for leptin or its receptor, or who exhibit subnormal secretion of the hormone, ruling out its current use as a generic drug. In most cases, obese humans have elevated leptin levels, indicative of leptin resistance. Unraveling the molecular mechanisms underlying this leptin resistance is therefore of great clinical interest. Given the complexity of the physiological processes controlling body weight, many defects may underlie leptin resistance. One possibility is a defect in the passage of leptin through the blood–brain barrier. It was suggested that the LR short isoform plays a role in this transport, but the precise mechanism is still a matter of debate and some evidence points to a hitherto uncharacterized leptin transporter in the brain capillary endothelium. Also, leptin resistance might result from a defect at the level of LR activation in the hypothalamic nuclei causing inappropriate sensing of the leptin levels. A better understanding of LR activation may help in understanding this, and may also provide a molecular explanation for the relative insensitivity of LR signaling in the presence of excess dominant negative LRsh receptors. Defects in LR signal transduction could be important as well, and in this light, negative regulators of signaling like SOCS3 and PTP1B are of special interest and may represent targets for the treatment of human obesity.

Acknowledgements: L.Z. and D.L. are research associate with the IWT and FWO-V, respectively. We apologize for those papers that have not been cited due to space restrictions.

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Chapter 7: Modulation of leptin receptor signalling

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.

Introduction

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 also in the stomach and in some other tissues [1;2]. Leptin is released in 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 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 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 JAK (janus kinase) kinases in 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 cytoplasmic 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 a YXXQ 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, indicatory for 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 MAPK (mitogen activated protein kinase) pathway (see below) and the PI3K (phosphoinositol 3-kinase) 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 phosphatidylinositol$_{4,5}$-biphosphate (PIP_2) into phosphatidylinositol$_{3,4,5}$-triphosphate (PIP_3) 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].

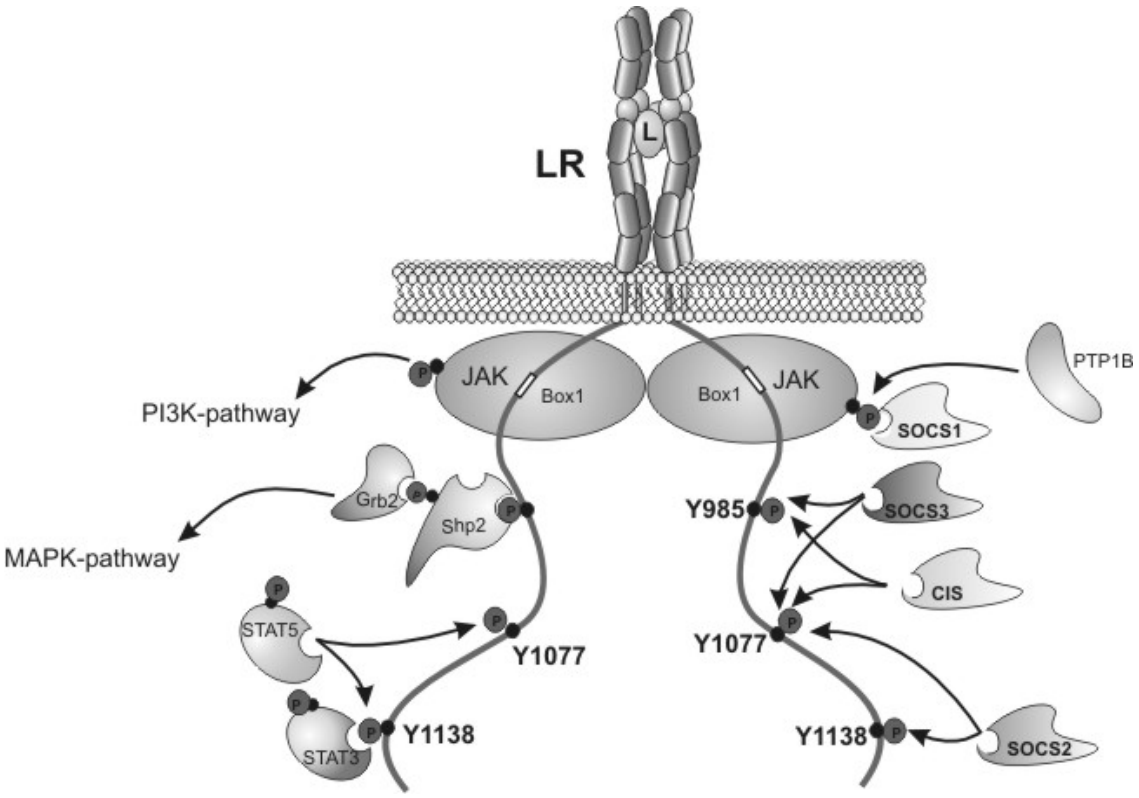

Figure 1: Schematic representation of LR signalling and negative regulation.
For abbreviations, we refer to the main text.

Modulation of functional receptor expression

Obviously, receptor internalisation is an effective mechanism to rapidly turn off cytokine signalling. Upon ligand binding cytokine receptors can be internalized 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 is found in intracellular pools [21].This distribution of the LR may be explained by its liability to constitutive endocytosis resulting in short-lived membrane expression. In addition, part 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}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 of the membrane [23]. Compared with other LR splice variants, the long LR isoform seemed 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 ubiquitinylated. 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 either protect their ligands from degradation or clearance and thus significantly extend their half-life or 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 and 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

SHP-2 (SH2 domain containing phosphatase-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 (cfr. infra), it also appeared as a strong activator of the MAPK pathway. ERK activation occurs predominantly via SHP-2 recruitment at 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 also attributed an inhibitory role in LR signalling to the SHP-2 phosphatase. 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, SOCS3 (suppressor of cytokine signalling 3), 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.

PTP1B (protein tyrosine phosphatase 1B) 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 PTP-1B levels. Aberrant PTP1B activity is implicated in leptin resistance and PTP1B is currently 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 PTEN (phosphatase and tensin homologue deleted on chromosome ten) 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 POMC (proopiomelanocortin) neuropeptide results in an obese phenotype associated with leptin resistance [71].

Suppressors of cytokine signalling

The family of SOCS proteins consists of 8 members: CIS (cytokine inducible SH2 protein) and SOCS1 through SOCS7. SOCS proteins have a characteristic domain structure which is represented in figure 2. They carry a central SH2 domain, an N-terminal preSH2 domain with an ESS (extended SH2 subdomain) region and in some cases a KIR (kinase inhibitory region) domain and a C-terminal SOCS-box [72]. The N-terminal domain varies in length and composition while the SH2 domain and the SOCS-box are more conserved. They also carry one or two conserved tyrosines in the C-terminus of their SOCS-box. SOCS proteins can interfere with cytokine signalling at different levels. They can interact with phosphotyrosine motifs in activated cytokine receptor complexes by means of their SH2 domain, thereby hindering association of signalling molecules. The SOCS-box of SOCS proteins is identified as a key mediator in targeting associated proteins for proteasomal degradation. It associates with elonginB/C via its BC-box and takes part in a multi-protein complex that acts as an

E3 ligase known to link ubiquitin to the substrate. Finally, the kinase activity of the JAKs can be abolished through the KIR domain.

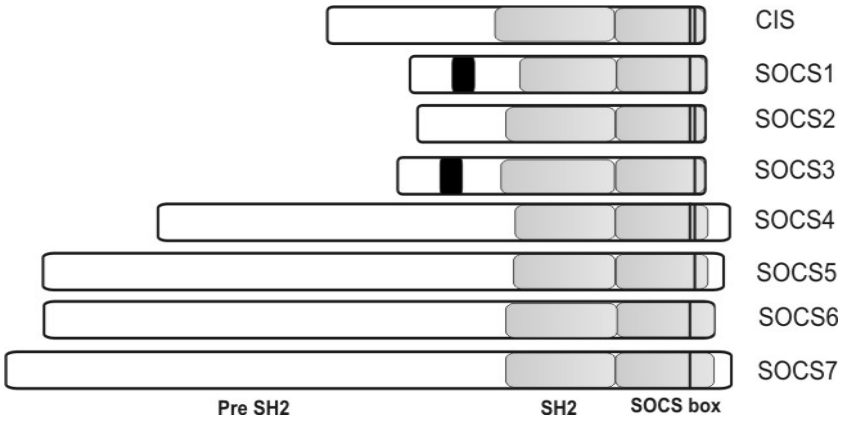

Figure 2: Schematic overview of SOCS protein structure.
The KIR domain is indicated with a black box, the C-terminal, conserved tyrosines are represented by a black line.

SOCS proteins are typically part of a negative feedback loop. They are induced upon cytokine stimulation and attenuate signalling by various cytokine receptors, allowing 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]. 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 administration in leptin-deficient ob/ob mice [12;43;76]. SOCS3 is a functional marker for identification of leptin-sensitive neurons 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 haploinsufficient or neural-cell specific deficient mice are viable and show augmented leptin sensitivity in the hypothalamus and a remarkable attenuation of diet-induced obesity [78;79]. It was demonstrated that SOCS3 action is involved in rendering the LR refractory to reactivation after chronic leptin stimulation [80]. These

observations put 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 phosphotyrosine 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].

	$_{P}$Y985		$_{P}$Y1077		$_{P}$Y1138
	MAPPIT	PAC	MAPPIT	PAC	PAC
CIS	+	-	+	-	-
SOCS1		-		-	-
SOCS2	-	-	+	+	+
SOCS3	+	+	- /+	+	-

Table 1: 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].

Using the MAPPIT technique, a two hybrid method based on cytokine signalling, we recently demonstrated the interaction 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 associated with the latter of the two. Phosphotyrosine specific interaction of SOCS2

with the LR Y1077 motif was confirmed 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) interactions 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 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 knock-outs and CIS transgenes 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 pro-inflammatory 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 be in part 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 like 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, since 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] (Piessevaux et al., in press). 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 (Piessevaux et al., in press). SOCS2 is demonstrated to associate with all members of the SOCS protein family [74;96] (Piessevaux et al., in press). 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] (Piessevaux et al., in press). 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] (Piessevaux et al., in press). 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 (Piessevaux et al., in press). 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 seen for other SOCS proteins [96-99].

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 [100]. It was reported that SOCS7 is implicated in LR signalling termination. It can inhibit STAT3 activation which we speculate may involve LR association but can also interact with activated STAT3 molecules to prevent them from translocating to the nucleus [101].

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 PTP-1B 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 signalling in many vital processes.

Acknowledgements

We greatly acknowledge Prof. Joël Vandekerckhove for continued support. This work was supported by grants from the Flanders Institute of Science and Technology (GBOU 010090 grant), from The Fund for Scientific Research – Flanders (FWO-V Grant N° 1.5.446.98) and from Ghent University (GOA 12051401).

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partIII

The role of SOCS proteins in LR signalling modulation

Scope of the thesis

Leptin plays a major role in regulation of energy homeostasis and food intake. It operates essentially as an adipostat whereby it communicates the status of body fat reserves to the central nervous system.

The leptin receptor predominantly activates the JAK-STAT pathway via JAK2 and STAT3. SOCS3 and PTP1B are identified as negative regulators of LR signalling. The phosphotyrosine phosphatase, PTP1B, primarily dephosphorylates JAK2, whereas SOCS3 is thought to predominantly inhibit JAK2 activity by means of its kinase inhibitory region. The importance of tight negative regulation is underscored by the state of relative leptin resistance observed in most cases of human obesity which is partly caused by defects in hypothalamic leptin signalling including aberrant signalling attenuation. In this work we focus on the role of SOCS proteins in the mediation of leptin receptor signal transduction in an attempt to elucidate the underlying mechanisms that involve the functionality of SOCS proteins in the leptin receptor signalling context.

We made extensive use of the versatile MAPPIT technique described in chapter 4. This technique is very well suited for examining signal transduction pathways. It is capable of identifying associations that are short-lived or that depend on tyrosine phosphorylation. We adapted the technique to allow the detection of interactions with the LR itself. Therefore we mutated the STAT3 recruiting tyrosine to phenylalanine. In a second adaptation, called GGS-MAPPIT, the intracellular part of the LR following the docking site of the JAK kinase was replaced by 60 GGS triplet repeats. This MAPPIT variant allows interaction with isolated tyrosine motifs of the LR and also avoids interfering interactions due to background association with the LR.

First, we investigated the binding properties of two members of the SOCS family, CIS and SOCS2, that we identified as new interaction partners of the LR. Their differential binding was examined using the tyrosine to phenylalanine LR mutants in the LR-MAPPIT technique, the GGS-MAPPIT method and LR-MAPPIT based competition binding assays. Both interactions were also examined by peptide affinity chromatography, a well established, biochemical approach. We further investigated the functionality of SOCS2 in the context of leptin receptor signalling (Chapter 8).

Secondly, we addressed the role of conserved tyrosines in the C-terminus of the SOCS-box of the four most thoroughly studied SOCS proteins namely CIS, SOCS1, SOCS2 and SOCS3. An array of mutants based on the particular tyrosines was tested for associating capacities and functionality in LR and EpoR signalling (Chapter 9).

In several cytokine receptor systems including the GHR signalling it was reported that SOCS2 could both inhibit and enhance signalling. This dual effect of SOCS2 led to the speculation of SOCS2 interfering with other SOCS proteins. We investigated and corroborated this hypothesis in the context of LR signalling and other cytokine systems. We used reporter assays and LR-MAPPIT assays to examine interference of SOCS2 with other SOCS proteins in leptin, growth hormone and IFN-γ signalling context. Degradation experiments and MAPPIT assays were applied to study the underlying mechanism of this interference phenomenon. Similar experiments were used to examine whether other SOCS proteins exerted comparable interfering characteristics (Chapter 8 and Chapter 10).

Chapter 8: A complex interaction pattern of CIS and SOCS2 with the leptin receptor.

In this article we report the differential binding of CIS and SOCS2 with the LR. We used the LR-MAPPIT technique described in chapter 4 with the LR itself deprived of its membrane distal STAT3 recruiting tyrosine as bait protein. One or both of the two membrane proximal tyrosines at positions Y985 and Y1077 were mutated to phenylalanine to examine tyrosine-specific interactions. We demonstrated association of both CIS and SOCS2 with the Y1077 position whilst CIS but not SOCS2 associated with the membrane proximal Y985 position. We next performed MAPPIT competition experiments and peptide affinity chromatography to investigate the binding properties of both SOCS proteins. CIS and SOCS2 are typically known as inhibitors of STAT5 activity as readily demonstrated in for example GHR signalling (Greenhalgh et al., 2002; Ram et al., 1999). We revealed here that SOCS2 can interfere with association of the SH2 domain of STAT5a at the Y1077 position. Furthermore, we demonstrated that SOCS2 interfered with CIS binding at the membrane proximal tyrosine in the LR, although SOCS2 itself did not interact with this position. Co-immunoprecipitation assays and MAPPIT interaction experiments subsequently showed that SOCS2 could associate with CIS, particularly with its SOCS-box. Finally, in analogy to a known elongin B/C recruitment-deficient SOCS1 mutant, we developed the SOCS2(LC-QQ) mutant. We demonstrated by TAP2 analysis, a two-step immunopurification method, that this SOCS2 variant was no longer capable of recruiting the elongin B/C complex to its SOCS-box. This SOCS2 mutant completely lost its regulatory capacity, suggesting that proteasomal degradation of CIS is involved.

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

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Accepted 20 February 2006
Journal of Cell Science 119, 2214-2224 Published by The Company of Biologists 2006
doi:10.1242/jcs.02947

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 (LRlo) 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 LR signalling 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

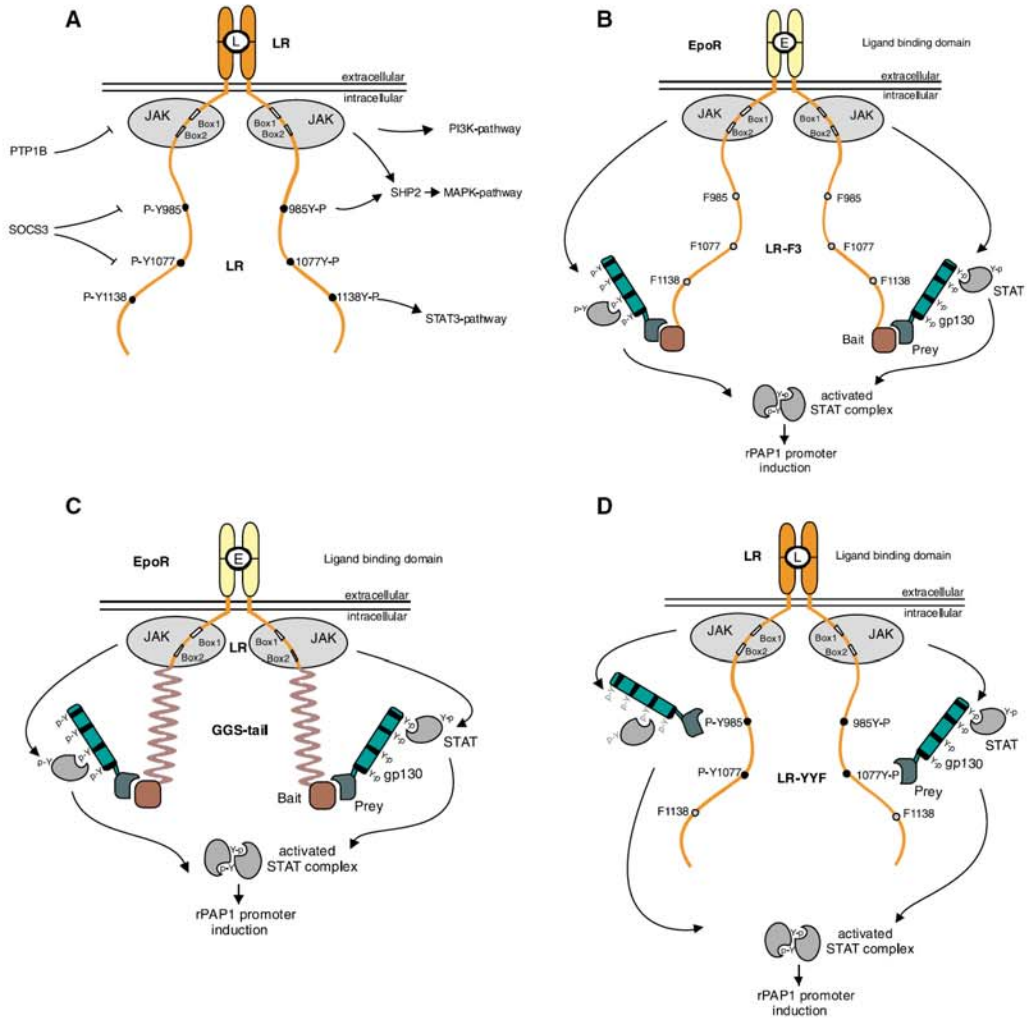

Fig. 1. (A) Overview of LR signalling and its interaction partners. The murine LR carries three conserved tyrosines in its cytoplasmic tail at positions Y985, Y1077 and Y1138. JAK2 is constitutively associated with the LR at the conserved Box 1 and 2 motifs. Upon leptin stimulation, the JAKs become fully 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 (Kile et al., 2002). Elongin B or C of the E3 ligase complex is recruited to the BC box in the SOCS box (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 hypothalamus 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 (Kaszubska 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 I (rPAPI) promoter-luciferase reporter (Fig. 1B). Intrinsic 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

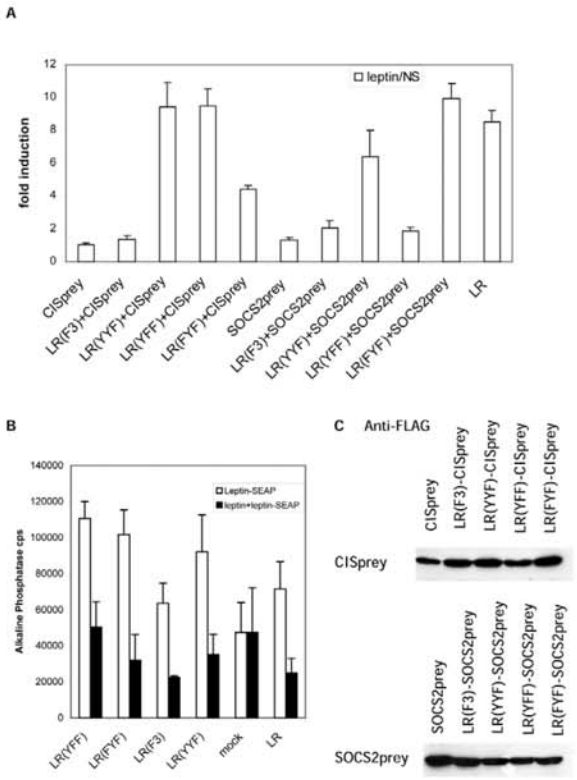

Fig. 2. Differential association of CIS and SOCS2 with the LR. (A) HEK293T cells were transiently co-transfected with plasmids encoding different pMET7-LR variants and the pMG2-CIS and pMG2-SOCS2 prey constructs, or with mock vector, combined with the pXP2d2-rPAP1-luci. The transfected cells were either 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) LR expression levels were measured on the same transfected cells by incubation for 2 hours with leptin-SEAP fusion protein with or without a 100-fold excess of unlabelled leptin. Mean bound SEAP activity + s.d. of triplicate measurements is plotted. (C) Western blot analysis of CISprey and SOCS2prey expression. Expression of the FLAG-tagged fusion prey proteins in the same transfected cells was verified on lysates using anti-FLAG antibody.

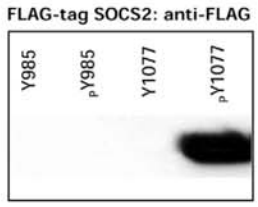

Fig. 3. SOCS2 interaction with the peptide matching the Y1077 motif of the LR is phosphorylation dependent. FLAG-tagged SOCS2 was expressed in HEK293T cells and lysates were incubated with phosphorylated or non-phosphorylated peptides corresponding to the Y1077 or Y985 motif. Immunoblotting with anti-FLAG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

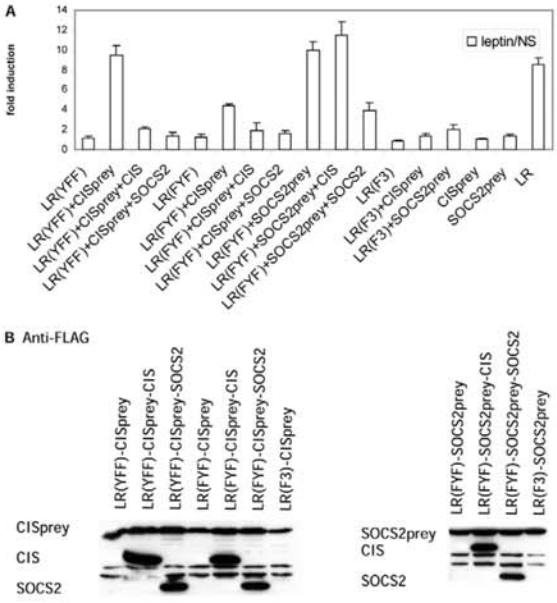

Fig. 4. Stability of the CIS and SOCS2 interactions with the LR. (A) HEK293T cells were transiently co-transfected with plasmids encoding different pMET7-LR variants, the pMG2-CIS or pMG2-SOCS2 prey construct, pEF-FLAG-I/mCIS or pEF-FLAG-I/mSOCS2, or the appropriate amount of mock vector together with 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.

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

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

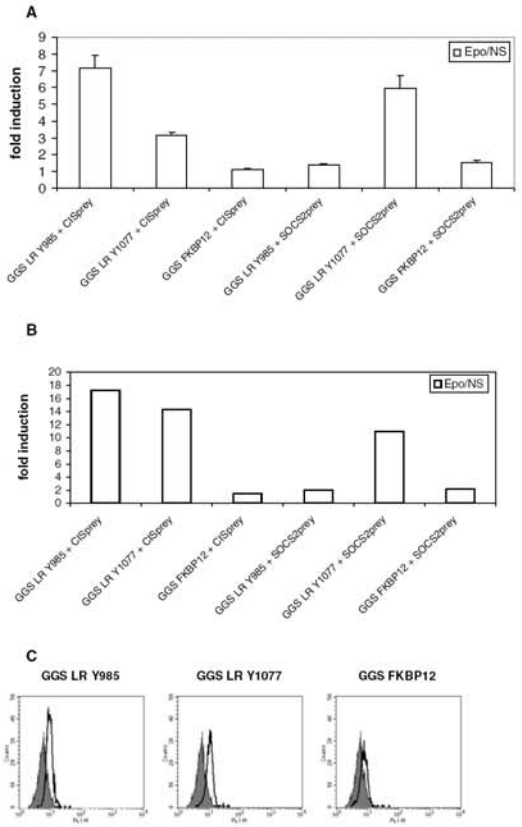

Fig. 5. GGS-MAPPIT analysis of CIS and SOCS2 interactions with the LR. (A,B) HEK293T cells (A) or TF-1 cells (B) were transiently co-transfected with plasmids encoding the chimeric bait constructs with the different LR motifs or with the FKBP12 control bait, and the pMG2-CIS, pMG2-SOCS2 prey constructs, 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.e.m. (C) FACS analysis shows the expression of the different chimeric GGS bait receptors in TF-1 cells. The grey filled curves represent the parental TF-1 cells; open lines, the transiently transfected TF-1 cells.

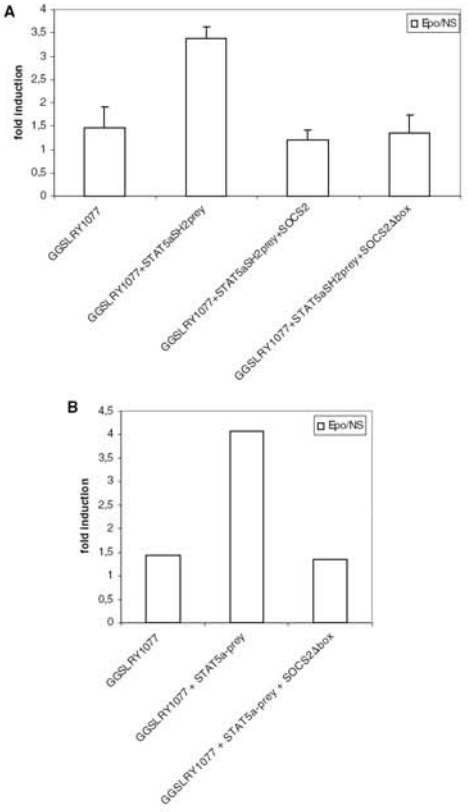

Fig. 6. SOCS2 interferes with the association of a STAT5a prey at Y1077. (A,B) HEK293T cells (A) or TF-1 cells (B) were transiently co-transfected with the plasmid encoding the GGS bait construct with the Y1077 LR motif, the pMG2-STAT5aSH2 prey construct, the pMET7-FLAG-SOCS2 or pMET7-FLAG-SOCS2 Δbox, 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 (Epo stimulated/NS) + s.d.

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).

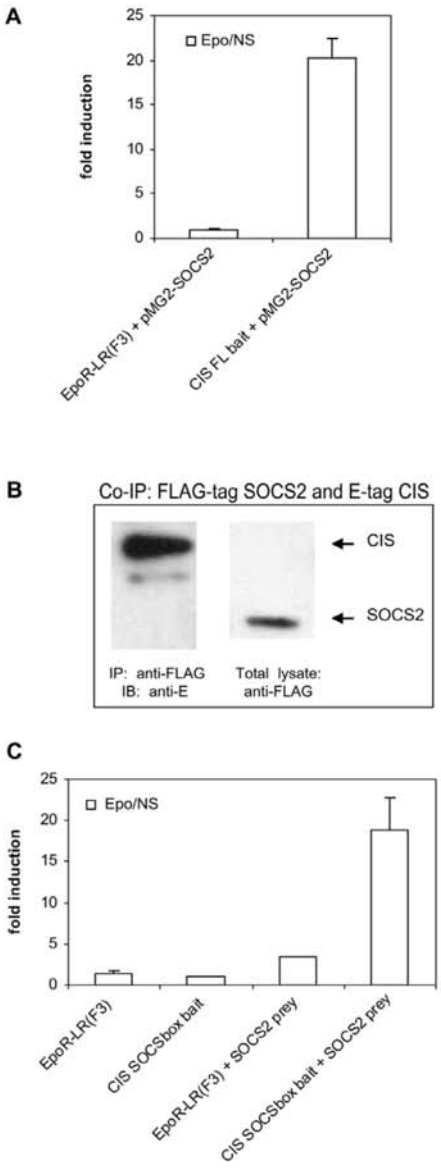

Fig. 7. SOCS2 interacts with CIS. (A) MAPPIT analysis. HEK293T cells were transiently co-transfected with plasmids encoding the chimeric EpoR-LR(F3) construct as a negative control or with the full-length (FL) CIS bait, and the pMG2-SOCS2 prey constructs, combined with the pXP2d2-rPAP1-luci reporter. 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. (B) Co-immunoprecipitation. HEK293T cells were transiently co-transfected with pMET7-Flag-SOCS2 and pMET7-Etag-CIS. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E. (C) SOCS2 interacts with the SOCS box of 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 prey construct or the appropriate amount of mock vector, 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.

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

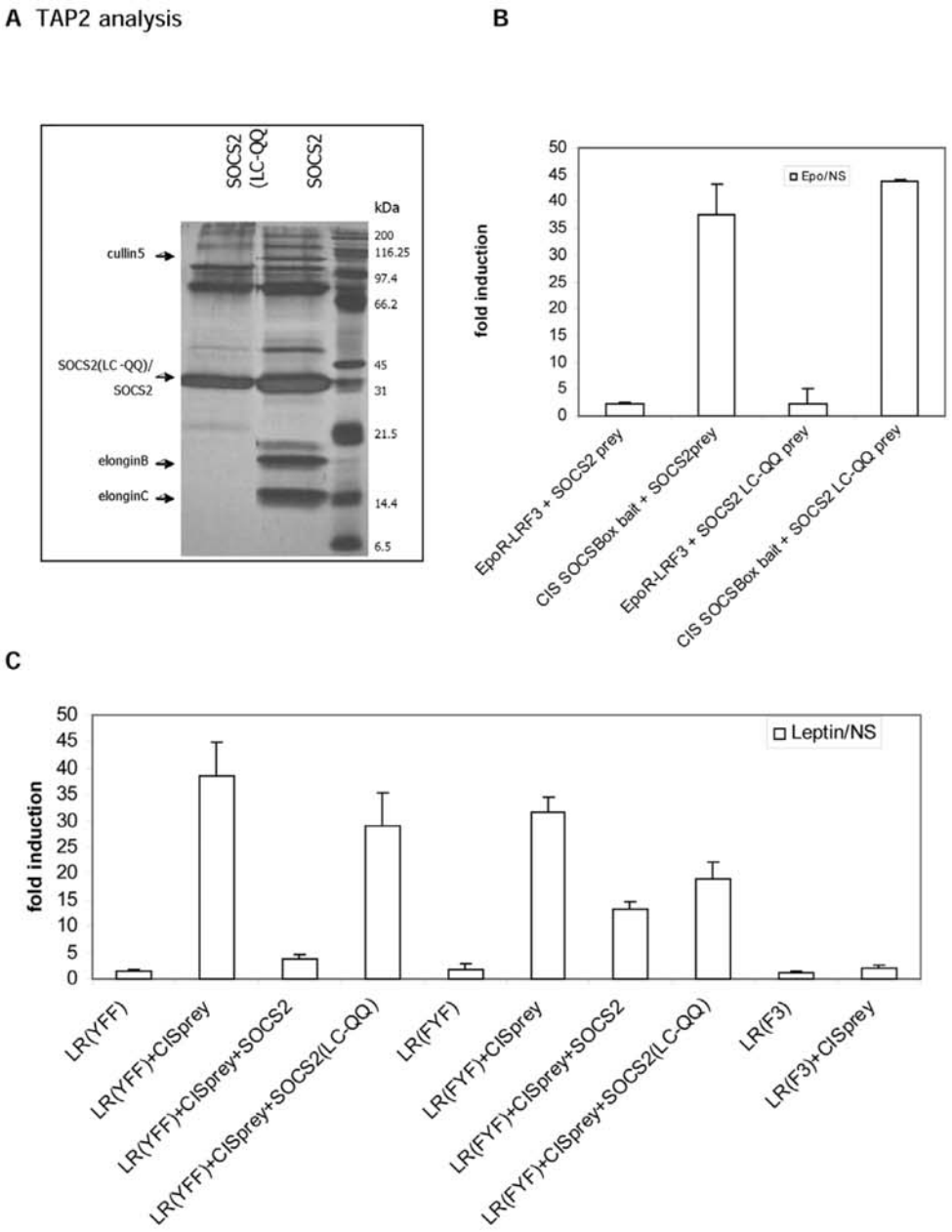

Fig. 8. (A) Generation of a SOCS2 mutant deficient in elongin B/C binding. HEK293T cells were transiently transfected with the pMET7TAP2-SOCS2 and pMET7TAP2-SOCS2(LC-QQ) constructs. Cell lysates were purified using the TAP2 tag and loaded on a polyacrylamide gel and silverstained. From a parallel experiment, 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 over-expression could clearly interfere with CISprey binding to either motif, no cross-competition with the SOCS2prey occurred. Conversely, SOCS2 could easily interfere with CISprey 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-modulation 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 shifts to 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,

since it is activated in different cell types upon leptin stimulation in vitro through the Y1077 and Y1138 positions in the LR (Baumann et al., 1996; Hekerman et al., 2005). We here showed that SOCS2 can inhibit STAT5a prey association at the LR Y1077 position.

In conclusion, the MAPPIT approach was shown to be a sensitive and flexible system for analysing interactions between proteins in a cellular context. We identified two SOCS proteins, CIS and SOCS2, as new interaction partners of the LR, and identified a novel regulatory role for SOCS2. Full understanding of the biological implications of cross-regulation between SOCS proteins on the different cytokine receptor systems will require detailed, quantitative analysis of all members of this protein family.

Materials and Methods

Constructs

Generation of the mutant mouse LR(YYF), LR(FYF), LR(YFF), LR(F3) in the pMET7 expression vector was described elsewhere (Eyckerman et al., 1999). A pSEL-hEpoR-Y480 bait vector was derived from the described pSEL-hEpoR-Y402 bait (Eyckerman et al., 2001). In this pSEL-hEpoR-Y480 bait construct most of the intracellular part of the LR was replaced by a flexible GGS_N linker. An unique *Eco*RI restriction site was introduced immediately following the JAK2 binding site through site-directed mutagenesis (Stratagene) using the primer pair 5′-GCTT-GGAAAAATAAAGATGAATTCGTCCCAGCAGCTATGGTC-3′ and 5′-GACC-ATAGCTGCTGGGACGAATTCATCTTTATTTTTCCAAGC-3′. Phosphorylated oligonucleotides encoding two GGS repeats (5′-TCTGGTGGCAGTGGAGGG-3′ and 5′-AGACCCTCCACTGCCACC-3′) were annealed and head to tail ligated. Ligation was stopped by addition of two other annealed oligonucleotide couples: 5′-AATTCGGAGGGAGTGGTGGC-3′ and 5′-AGAGCCACCACTCCCTCCG-3′; 5′-TCTGGAGGGAGTGGTGGGAGCT-3′ and 5′-CCCACCACTCCCTCC-3′. Annealing of these oligonucleotide couples generated respectively an *Eco*RI and a *Sac*II (both underlined) sticky end. The final product was ligated in *Eco*RI-*Sac*II opened pSEL-hEpoR-Y480 vector. Sequence analysis showed that using this procedure 20 GGS repeats were introduced in the bait construct. The linker was further amplified using oligonucletides 5′-CTTCTTCTGGAGCCTGAACC-3′ and 5′-CGCCGCCAATTGCGAACTCCCACCACTCCC-3′. The product was digested with *Eco*RI-*Mfe*I and ligated in the *Eco*RI digested pSEL-20GGS-hEpoR-Y480 vector yielding the pSEL-40GGS-hEpoR-Y480 vector. This ligation step was repeated once more to generate pSEL-60GGS-hEpoR-Y480 vector. The pSEL(+2L)60GGS-Y480 construct was generated by site-directed mutagenesis on the pSEL-60GGS-hEpoR-Y480 vector using primers 5′-CCCATAATTATTTCC-AGCTGTCTCCTCGTCCTACTGCTCGGAAC-3′ and 5′-GTTCCGAGCAGTAG-GACGAGGAGACAGCTGGAAATAATTATGGG-3′ and Y480 was removed by *Sac*I-*Not*I. The mLR Y985 motif was amplified with forward primer 5′-GCCGAGCTCATGGAAAAATAAAGATGAG-3′ containing a *Sac*I site and reverse primer 5′-CGGGCGGCCGCTCAACAGACAGACTTCTCCCTGTG-3′ containing a *Not*I site and a stop codon, allowing in-frame coupling to the hEpoR-60GGS chimeric receptor. The same strategy was used for the mLR Y1077 motif, using primers 5′-GCCGAGCTCAGCAACTCTGGTCAGCAAC-3′ and 5′-GGG-CGGCCGCTCAAGGTACAAAGTTCTCACC-3′. The final constructs were called pSEL(+2L)60GGS-mLR-Y985 and pSEL(+2L)60GGS-mLR Y1077, respectively. For the pSEL(+2L)60GGS-FKBP12 construct, FKPB12 was cut from the pSELFFY-FKBP12 construct described earlier (Eyckerman et al., 2005) using *Sac*I and *Not*I, and cloned in the pSEL(+2L)60GGS vector.

The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis-associated protein I) promoter, is used as previously described (Eyckerman et al., 2001). Expression plasmid vectors pEF-FLAG-I/mSOCS2 and pEF-FLAG-I/mCIS were a gift from R. Starr (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). Generation of the prey constructs pMG2-CIS and pMG2-SOCS2 both containing part of the gp130 chain (aa 905-918) in duplicate was described elsewhere (Montoye et al., 2005).

The pMET7-FLAG-CIS construct was created by a three-point ligation. The N-terminal FLAG-tag of CIS was cut from the pEF-FLAG-I/mCIS construct using *Eco*RI-*Pvu*II and the C-terminal part of CIS was cut from the pMG1-CIS construct using *Kpn*I-*Pvu*II as described elsewhere (Eyckerman et al., 2001). Both parts were ligated in the pMet7 vector digested with *Eco*RI-*Pvu*II. To create the pMET7-Etag-CIS construct, CIS was amplified from the pMET7-FLAG-CIS using primers 5′-CGTCCGCGGCCGCGGTCCTCTGCGTACAGGGATC-3′ and 5′-GCTGGCT-CGAGTCAGAGTTGGAAGGGGTACTGTC-3′ and was ligated in the *Not*I-*Xho*I digested pCAGGSE-mMyD88 construct, which was a gift from Rudy Beyaert (Ghent University, Ghent, Belgium). Etag-CIS was then digested with *Eco*RI-*Xho*I and ligated in the pMET7 vector.

pMET7-FLAG-SOCS2 was created by cutting SOCS2 from the pMG2-SOCS2 construct using *Eco*RI-*Xba*I and ligating it into the *Eco*RI-*Xba*I digested pMET7-FLAG-SOCS3 expression vector which was described elsewhere (Eyckerman et al., 2000). SOCS2 Δbox was amplified using primers 5′-TGCCTTTACTTCTAGGC-CTG-3′ and 5′-GCAGGTCTAGATTATGATGTATACAGAGGTTTG-3′ from the templates pMET7-FLAG-SOCS2 cloned in the *Not*I-*Xba*I opened pMET7-FLAG-SOCS2 to create pMET7-FLAG-SOCS2 Δbox. The pMET7-FLAG-SOCS2(LC-QQ) mutant was created by site-directed mutagenesis of the pMET7-FLAG-SOCS2 construct using primers 5′-GTATACATCAGCACCCACTCAGCAGCATTTCCA-ACGACTCGCCATTAAC-3′ and 5′-GTTAATGGCGAGTCGTTGGAAATGCTG-CTGAGTGGGTGCTGATGTATAC-3′.

Generation of the chimeric receptors containing the extracellular part of the EpoR and the transmembrane and intracellular parts of the leptin receptor, such as EpoR-LR(F3), were described elsewhere (Eyckerman et al., 2001). A full-length CIS bait construct was generated by digesting the pCEL(2L)-Y480 bait construct with *Sst*I and *Not*I and swapping the EpoR Y402 motif with a PCR product containing full-length CIS and the cloning sites *Sst*I and *Not*I (primers 5′-GCGCGAGCTC-AATGGTCCTCTGCGTACAGGG-3′ and 5′-GCTCGCGGCCGCTCAGAGTTG-GAAGGGGTACTGTCGG-3′). The CIS SOCS box bait was made using the same strategy and the PCR amplification of the CIS SOCS box was done with primers 5′ GCGAGAGCTCCGGATCCGCCCGCAGCTTACAACATC and 5′ CGCTG-CGGCCGCTTAGAGTTGGAAGGGGTACTG. The SOCS2 (LC-QQ) prey was generated by mutating L163 and C167 of the Wild Type SOCS2 prey using primers 5′-GTATACATCAGCACCCACTCAGCAGCATTTCCAACGACTCGCCATTAAC-3′ and 5′-GTTAATGGCGAGTCGTTGGAAATGCTGCTGAGTGGGTGCTG-ATGTATAC-3′. pMG2-STAT5aSH2 was created by amplifying the SH2 domain of STAT5a using primers 5′-GCGAGAATTCTCCGGACCCCACTGGAATGATGG-GGC-3′ and 5′-CGCTTCTAGATTAACTCGAGGAGAAGACCTCATCCTTGG-3′ and *Eco*RI-*Xba*I cloning in the pMG2 vector.

To generate the pMET7TAP2 construct we used the primers 5′-GCGAG-GGCCCGCCACCATGGCCCAGCACGACGAGATCTC-3′ and 5′-CGCTCTCG-AGGCCCTGGAAGTACAGGTTCTCGCTGGCGGTGGTGGGGATGTCGCTGT-TGGCGTCCACGCTG-3′ to amplify the proteinA domain from the pMA-*Spa*I construct obtained from the BCCM/LMBP plasmid collection and to add a TEV cleavage site and we cloned the PCR product in the pMET7 vector using *Apa*I-*Xho*I. The FLAGtag was introduced using primers 5′-GCGAGGGCCCGCCACCAT-GGCCCAGCACGACGAGATCTC-3′ and 5′-GCGAGAATTCCCCGCTGCCCT-TGTCATCGTCGTCCTTGTAGTCCTGGCGCGCGCCCTGGAAGTACAGGTTC-3′ and cloned in the same vector using *Apa*I-*Eco*RI.

pMET7TAP2-SOCS2 was constructed by cutting SOCS2 from the pMG2-SOCS2 construct using *Eco*RI-*Not*I and ligating it in the pMET7TAP2 construct.

This construct was then used to create the pMET7TAP2-SOCS2(LC-QQ) by site-directed mutagenesis using the primers 5′-GTATACATCAGCACCCACTCAGC-AGCATTTCCAACGACTCGCCATTAAC-3′ and 5′-GTTAATGGCGAGTCGTT-GGAAATGCTGCTGAGTGGGTGCTGATGTATAC-3′. All constructs were verified by DNA sequence analysis.

Cell culture, transfection and reporter assays

Culture conditions, transfection procedures and luciferase assays for HEK293T cells were as previously described (Eyckerman et al., 2000). For a typical luciferase experiment, HEK293T cells were seeded in six-well plates 24 hours before overnight transfection with the desired constructs together with the luciferase reporter gene. Two days after transfection cells were left untreated (not stimulated NS) or were stimulated with 100 ng/ml leptin overnight. The luciferase activity of the transfected cells were measured by chemiluminescence. The factor-dependent TF-1 erythroleukaemia cell line was grown in RPMI medium supplemented with 10% foetal calf serum and 1 ng/ml GM-CSF. After electroporation (300 V, 1500 μF), cells were starved (removal of GM-CSF) for 24 hours and were subsequently stimulated with 5 ng/ml hEpo overnight. After 24 hours, luciferase activity was measured as described before.

Leptin binding assay

LR expression on the surface of HEK293T cells was measured using a binding assay with a mouse leptin-secreted alkaline phosphatase (SEAP) chimeric protein (Tartaglia et al., 1995). 48 hours after transfection, cells were incubated for 2 hours with a 1:50 dilution of conditioned Cos1 medium containing the chimeric protein with or without an excess of leptin. After two washing steps, cells were lysed in a buffer (1% Triton X-100, 20 mM Tris-HCl pH 7.4) and alkaline phosphatase activity was measured by chemiluminescence, using CSPD substrate (PhosphaLight, Tropix) according to the protocol provided by the manufacturer.

FACS analysis

The expression of the chimeric hEpoR-mLR GGS baits was monitored using goat anti-human EpoR polyclonal IgG (R&D Systems) at 2 μg/ml and Alexa Fluor 488-conjugated donkey anti-goat IgG (Molecular Probes) at 4 μg/ml. Fluorescence-activated cell sorting (FACS) was performed on a FACSCalibur (Becton Dickinson).

Western blot analysis

Expression of the gp130-fusion proteins, CIS and SOCS2, all flag-tagged, were

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, 0.5% DOC, Complete™ Protease Inhibitor Cocktail (Roche). 4× 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) (Amersham Biosciences).

(Phospho)peptide affinity chromatography

Approximately 35×10^6 HEK293T cells were transfected with either pEF-FLAG-1/mSOCS2 or pEF-FLAG-1/mCIS and were lysed in lysis buffer (20 mM HEPES pH 7, 1 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 150 mM NaCl, 0.5% NP40, 20% glycerol, 1 mM $NaVO_4$, 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 2× 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$_{(P)}$Y$_{985}$ATLVSNDK and biotin-NHREKSVC$_{(P)}$Y$_{1077}$LGVTSVNR. Synthesis and purification of the biotinylated (phospho)tyrosine peptides and coupling to streptavidin-agarose beads was described before (Eyckerman et al., 2000).

Co-immunoprecipitation

Approximately 2×10^6 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 (Amersham Biosciences). After immunoprecipitation, SDS-PAGE and western blotting, interactions were detected using anti-E-Tag antibody (Amersham Biosciences).

TAP2 purification and mass spectrometry

HEK293T cells were transfected with the appropriate constructs and lysed in cell lysis buffer (50 mM Tris-HCl pH 8, 10% glycerol, 1% NP40, 150 mM NaCl, 5 mM NaF, 5 μM $ZnCl_2$, 1 mM Na_3VO_4, 10 mM EGTA, Complete™ Protease Inhibitor Cocktail). The insoluble fraction was centrifuged and the supernatant was incubated with IgG Sepharose (Amersham Biosciences) overnight. The beads were washed three times with washing buffer (20 mM Tris-HCl pH 7.5, 5% glycerol, 0.1% NP40, 150 mM NaCl) and twice with TEV (Tobacco Etch Virus) protease cleavage buffer 1 (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA) and were then incubated with TEV protease in TEV protease cleavage buffer 1 for 2 to 4 hours. The beads were then centrifuged and the supernatant was incubated with anti-FLAG agarose (Sigma) in TEV protease cleavage buffer 2 (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.1% NP40) for 2 to 4 hours. The anti-FLAG agarose beads were washed three times with washing buffer and incubated with FLAG peptide for 10 minutes. The beads were spun down and 4× loading buffer was added to the supernatants before loading on a polyacrylamide gel. Proteins were either visualised by silver staining, or for mass spectrometry analysis with Sypro Ruby protein gel stain according to the manufacturer's instructions (Molecular Probes). Proteins of interest were excised and in-gel digested with trypsin as described. The resulting peptide mixture was dried, re-dissolved in 20 μl of 0.1% formic acid in 2/98 (v/v) acetonitrile/water and half of it was applied for nano-LC-MS/MS analysis on an Ultimate (Dionex, Amsterdam, The Netherlands) in-line connected to an Esquire HCT ion trap (Bruker Daltonics, Bremen, Germany). The sample was first trapped on a trapping column (PepMap™ C18 column, 0.3 mm ID ×5 mm, Dionex) and after back-flushing, the sample was loaded on a 75 μm ID ×150 mm reverse-phase column (PepMap™ C18, Dionex). The peptides were eluted with a linear solvent gradient over 50 minutes of 0.1% formic acid in acetonitrile/water (7/3, v/v). Using data-dependent acquisition, only multiple charged ions with intensities above threshold 100,000 were selected for fragmentation. For MS/MS analysis, an MS/MS fragmentation amplitude of 0.7 V and a scan time of 40 milliseconds was used. The fragmentation spectra were converted to Mascot generic files (mgf) using the Automation Engine software (version 3.2, Bruker) and searched using the MASCOT database search engine (http://www.matrixscience.com) against the SwissProt and the NCBInr Database (taxonomy mammalia). Only spectra that exceeded Mascot's threshold score for identify (set at the 95% confidence level) were retained for further manual validation.

We greatly acknowledge the technical support from Delphine Defeau 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 (GBOU 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 12051401) and from the Deutsche Forschungsgemeinschaft (SFB 542 to W.B.).

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Chapter 9: The C-terminus of CIS defines its interaction pattern.

In this report we examined the interaction pattern of the most commonly studied SOCS proteins namely CIS, SOCS1, SOCS2 and SOCS3, in more detail. In the C-terminus of the SOCS-box these particular SOCS proteins carry one or two conserved tyrosines. MAPPIT analysis demonstrated that not only the SH2 domain but also the SOCS-box is indispensable for association of CIS with the LR. Site-directed mutagenesis revealed that the C-terminal tyrosine at position Y253 in the SOCS-box of CIS is critical for receptor binding. Both MAPPIT and peptide affinity chromatography experiments confirmed that the C-terminal tyrosine was also essential for interaction with the Y402 domain in the EpoR. Mutation of this tyrosine also abrogated the inhibitory action of CIS on EpoR signalling. In contrast, this phenomenon was not observed for interaction of CIS with the unrelated MyD88 protein, a universal adaptor protein in TLR signalling.

Conversely, the SOCS-box of the other examined SOCS proteins was not involved in association with the cytokine receptor motifs that were studied.

THE C-TERMINUS OF CIS DEFINES ITS INTERACTION PATTERN

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In press, Biochemical Journal

Summary

Proteins of the Suppressors of Cytokine Signaling (SOCS) family are characterised by a conserved modular structure with pre-SH2, SH2 and SOCS-box domains. Several members including CIS, SOCS1 and SOCS3 are rapidly induced upon cytokine receptor activation and function in a negative feedback loop, attenuating signalling at the receptor level. We used a recently developed mammalian two-hybrid system (MAPPIT) to analyse SOCS protein interaction patterns in intact cells, allowing direct comparison with biological function. We find that, besides the SH2 domain, the C-terminal part of the CIS SOCS-box is required for functional interaction with the cytokine receptor motifs examined, but not with the N-terminal Death Domain of the TLR adaptor MyD88. Mutagenesis revealed that one single tyrosine at position 253 is a critical binding determinant. Much in contrast, substrate binding by the highly related SOCS2 protein, and also by SOCS1 and SOCS3, does not require their SOCS-box.

Introduction

A wide spectrum of α-helical bundle cytokines contributes to growth, differentiation and survival of hematopoietic cells. Examples include the colony-stimulating factors (CSFs), erythropoietin (Epo) and several interleukins (ILs) such as IL-5. More recently, also leptin, a hormone-like member of this family, was shown to promote proliferation of hematopoietic progenitors [1-3]. All these cytokines activate the highly conserved JAK/STAT signalling pathway upon receptor binding. Signalling via these receptors is under tight control including negative feedback by rapidly induced

SOCS proteins. CIS was the founding member of the SOCS protein family that consists of 8 members: SOCS1-7 and CIS [4,5]. All SOCS proteins comprise an SH2-domain responsible for association with phosphotyrosine motifs, an N-terminal preSH2-domain and a C-terminal SOCS-box [5]. They can modulate receptor activation and signalling via at least three distinct mechanisms.

CIS can inhibit Epo and growth hormone (GH) signalling by competition for STAT5 docking sites at the receptor level [6-8]. Consistent with this, CIS suppresses Epo-induced cell proliferation and promotes apoptosis of erythroid progenitor cells [9,10]. Phenotypes of CIS transgenic mice and of STAT5a and/or b knockout mice show clear similarities, lending further support for CIS as a specific negative feedback regulator of STAT5-mediated cytokine signalling [4,6,7,11]. Direct interference with STAT5 recruitment is also suggested for SOCS2-mediated inhibition of GH action [12,13]. Interestingly, SOCS2 shows a dual effect on GH signalling. Mice lacking SOCS2 and SOCS2 transgenic mice both exhibit increased growth [12,14,15]. This may be explained through direct binding and functional interference between SOCS proteins [16,17].

CIS-dependent receptor degradation was reported for the EpoR and GHR [6,18]. The SOCS-box of SOCS proteins can interact with the Elongins B and C [19], which form a complex with proteins of the Cullin and Rbx families. This assembly is a E3 ubiquitin ligase complex that is responsible for specific targeting of associated proteins for poly-ubiquitination [20]. This way, several SOCS proteins can inhibit signalling by marking associated signalling components for proteasomal degradation.

SOCS1 and -3 carry a KIR (kinase inhibitory region) domain in their N-terminal region that acts as a pseudo-substrate for direct inhibition of JAK kinase activity. SOCS1 interacts directly with the phosphorylated activation loop of JAK2 via its SH2 domain [21] while SOCS3 shows only weak affinity for JAK2 and is thought to bind to the receptor in close proximity of the kinase [22]. This is exemplified for SOCS3 that was recently identified as a potent inhibitor of LR signalling involved in regulation of energy balance. SOCS3 haplo-insufficient mice or neural cell-specific SOCS3 knockout mice show augmented leptin sensitivity in the hypothalamus associated with a remarkable attenuation of diet-induced obesity, suggesting a key role for SOCS3 in leptin resistance [23,24].

SOCS proteins are also involved in regulating JAK-STAT independent pathways such as insulin and TNF-α signalling [25-27]. Also, triggering of Toll-like receptors (TLRs), key players in innate immunity, leads to the induction of CIS, SOCS1 and SOCS3 [28-30]. Evidence linking SOCS proteins to TLR signalling arose from the analysis of SOCS1-deficient mice that show enhanced sensitivity to LPS-induced sepsis [31,32] and from SOCS1-deficient mice that lack endotoxin tolerance. Moreover, macrophages lacking SOCS1 produce increased levels of nitric oxide and pro-inflammatory cytokines in response to LPS. Conversely, SOCS1 over-expression in macrophages suppresses LPS-induced NF-κB activation. Based on these data, SOCS1 was prompted as a negative regulator of TLR signalling, although no direct target of SOCS1 could be identified. Recently, two groups reported that SOCS1 has an indirect inhibitory effect on TLR signalling, targeting the secondary type I IFN signalling pathway and not the main NF-κB pathway [30,33].

In this paper we examine the binding modalities of SOCS proteins in more detail. We demonstrate that the SOCS-box of CIS, and more particularly its C-terminal tyrosine, is essential for interaction with recruitment motifs in the EpoR and LR and for its inhibitory role on STAT5 activation. In contrast, the SOCS-box is not required for SOCS2 receptor interaction or for signalling inhibition by SOCS1 and SOCS3. Furthermore, we identified the universal TLR adaptor MyD88 as a target for CIS. This interaction is SOCS-box-independent, indicating a different binding modus compared to the cytokine receptors.

Experimental Procedures

Constructs

Generation of the mutant murine LRs by mutagenesis and cloning in the pMET7 expression vector was published elsewhere [34]. EpoR-bait constructs containing 2 extra leucines in the transmembrane were described before [35]. The pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter was used as previously described [36]. The pGL3-beta-casein-luciferase reporter consisting of 5 repeats of the STAT5-responsive motif of the β-casein promoter was a gift from Dr. Ivo Touw. Generation of the prey constructs pMG2-CIS and pMG2-SOCS2 was described before [35].

CISd5, CISdbox (aa 1-221) , CISY249F and CISY253F prey constructs were generated by site directed mutagenesis on pMG1-CIS using following primer pairs:
CISd5:
5'-GACTACCTCCGACAGTGATATCTCCAACTCTGATCTAG-3' and
5' CTAGATCAGAGTTGGAGATATCACTGTCGGAGGTAGTC-3',
CISdbox:
5'-GTGCGCAGGAGCAGTTGATATCGCTTACAACATCTGTG-3' and
5'-CACAGATGTTGTAAGCGATATCAACTGCTCCTGCGCAC-3',
CISY249F:
5'-GGCGTATGGCCGACTTCTTAAGACAGTACCCCTTCC-3' and
5'-GGAAGGGGTACTGTCTTAAGAAGTCGGCCATACGCC-3',
CISY253F:
5'-GACTACCTCCGACAGTTCCCCTTCCAGCTGTGATCTAGAGAAAAAACCTCC-3' and 5'-GGAGGTTTTTTCTCTAGATCACAGCTGGAAGGGGAACTGTCGGAGGTAGTC-3',
The CIS mutants were then transferred to the pMG2 vector and the pMET7-FLAG expression vector by EcoRI-XbaI cloning.
SOCS2Y149F was generated using the previously described pMG1-SOCS2 as template [36] and the primer set
5'-GCAGAATTCACCCTGCGGTGCCTGGAGCC-3' and
5'-CGCTGCGGCCGCTTATACCTGGAATTTGAATTCTTCCAAGTAATC-3'
and was first cloned in the pMG1 vector as an EcoRI-NotI fragment, and then transferred to the pMG2 vector using EcoRI-XbaI. SOCS2Y190F and SOCS2dbox (aa 1-159) were amplified from the pEF-FLAG-I/mSOCS2 construct (gift from Dr. Starr) using the
5'-GCAGAATTCACCCTGCGGTGCCTGGAGCC-3' and the
5'-
GGTCGTCTAGAGCGGCCGCTTATACCTGGAATTTATATTCTTCCAAGAAATCTTTTAGTCTTGTTG-3' and
5'-GCTGGGCGGCCGCTTATGATGTATACAGAGGTTTGG-3' primers respectively, and were also cloned in the pMG2 vector. SOCS2 was transferred from the pMG2 vector to the pMET7-FLAG expression vector as an EcoRI-XbaI or EcoRI-NotI fragment.
The pMET7-FLAG-SOCS3 expression vector was described elsewhere [37]. SOCS3dbox was amplified from the pMET7-FLAG-SOCS3 template using primers

5’-GCGAGATCTCAGAATTCGTCACCCACAGCAAGTTTCC-3’ and
5’-CGCTTCTAGATTAGTTGGAGGAGAGAGGTCGG-3’ allowing EcoRI-XbaI based cloning in the pMet7-FLAG vector.
The pMet7-FLAGmSOCS1 and pMet7-FLAGmSOCS1dbox constructs were generated by amplifying SOCS1 and SOCS1dbox from the pEF-FLAG-I/mSOCS1 construct (gift from Dr. Starr) with the
5’-CCAGCGAATTCATGGCGCGCCAGGACTACAAGGAC-3’ and
5’-GGTCGTCTAGATCAGATCTGGAAGGGGAAGGAAC-3’ or
5’-GGTCGTCTAGATCAGCGGCGCTGGCGCAGCGGGGCCCCCAAC-3’ primer sets, respectively and EcoRI-XbaI cloning in the pMet7-FLAG vector.
EpoR cDNA was amplified from TF1-derived using primers
5’-CGGGGTACCATGGACCACCTCGGGGCGTCC-‘3 and
5’- CGCTCTAGACTAAGAGCAAGCCACATAGC-3’ and was cloned in the pSVsport vector by KpnI-XbaI cloning. The pECE-STAT5B expression vector was a gift from Dr. Becker.
Mouse full-length MyD88 was amplified using the
5’-GCGCGAGCTCAATGTCTGCGGGAGACCCCCGCG-3’ and
5’-GCGTGCGGCCGCTCAGGGCAGGGACAAAGCC-3’ primer pair on a pCAGGSE-mMyD88 expression vector (gift from Dr. Beyaert). After SacI/NotI digestion, the fragment was cloned in the pCEL(2L) vector, which was described earlier [35]. This resulted in the mMyD88 bait construct. The mMyD88(N) and the mMyD88TIR bait vectors were made in an analogous manner using primer pairs
5’-GCGCGAGCTCAATGTCTGCGGGAGACCCCCG-3’ /
5’-GCTCGCGGCCGCTTACGTTTGTCCTAGGGGGTC-3’
and
5’-GCGCGAGCTCAATGCCGGAACTTTTCGATGCC-3’ /
5’-GCTCGCGGCCGCTCAGGGCAGGGACAAAGCC-3’ respectively.
The pCAGGSE-mMyD88(N) and pCAGGSEmMyD88 DD expression vectors were generated by amplification using oligo pairs
5’-GGCAAAGAATTGAATTCCACCATGGGTGCGC-3’ /
5’-GCGCCTCGAGTCAAAGTTCCGGCGTTTGTCCTAGGGGGTC-3’ and
5’-GGCAAAGAATTGAATTCCACCATGGGTGCGC-3’/

5'-GCGCCTCGAGTCAGACAGACGCGCCAGAGCGCCCCTGCC-3' respectively on pCAGGSE-mMyD88, followed by a EcoRI/XhoI digestion and ligation in the pCAGGSE vector.

1. Extracellular EpoR - Intracellular LRF3 (Y985F-Y1077F-Y1138F) bait constructs	
	Bait protein
Mock bait	No bait
EpoRY402 bait	Y402 motif of the EpoR
EpoRY430/Y432 bait	Y430/Y402 motif of the EpoR
mMyD88 bait	MyD88
mMyD88(N) bait	N-terminal part of MyD88 (Death Domain and intermediate domain)
mMyD88(TIR) bait	C-terminal TIR domain of MyD88
2. Extracellular LR – Intracellular LR bait constructs	
LR(F3)	LR (Y985F-Y1077F-Y1138F)
LR(YYF)	LR (Y1138F)
LR(YFF)	LR (Y1077F-Y1138F)
LR(FYF)	LR (Y985F-Y1138F)
3. Non-MAPPIT receptor constructs	
EpoR	WT EpoR
LR(FFY)	LR (Y985F-Y1077F)
LR(YFY)	LR (Y1077F)

Table 1: overview of the bait constructs used in this study

Generation of the pMET7TAP2 construct was described elsewhere [17]. CIS, CISdbox, CISY249F and CISY253F were introduced in a TAP2 construct via EcoRI-KpnI cloning from the respective pMG2 constructs.

An overview of the bait constructs used in this study is given in Table 1.

Cell culture, transfection and reporter assays

Cell culture conditions, transfection procedures and luciferase assays for Hek293T cells were previously described [37]. For a typical luciferase experiment, $4x10^5$ cells were seeded in 6-well plates 24 hours before transfecting them overnight with the desired constructs together with the luciferase reporter gene. Cells were left

untreated (negative control NC) or were stimulated overnight with 100 ng/ml leptin or 3.3 ng/ml Epo and luciferase activity of the transfected cells was measured by chemiluminescence.

Co-immunoprecipitation

Approximately 2 x 10^6 Hek293T cells were transfected with different combinations of mMyD88-E, mMyD88(N), mMyD88 DD, mCIS-FLAG, mCISY253F and mCISdBox. Cleared lysates (modified RIPA lysis buffer: 200mM NaCl, 50mM Tris-HCl pH8, 0,05% SDS, 2mM EDTA, 1% NP40, 0,5% DOC, Complete™ Protease Inhibitor Cocktail (Roche)) were incubated with 4 µg/ml anti-FLAG mouse monoclonal antibody (Sigma) and protein G-sepharose (Amersham Biosciences). After immunoprecipitation, SDS-PAGE and Western Blotting, interactions were detected using anti-E-Tag antibody (Amersham Biosciences) and anti-mouse-HRP (horseradish peroxidase) (Amersham Biosciences).

Phosphopeptide affinity chromatography

The phosphopeptide affinity chromatography procedure was as previously described [37].

Electrophoretic mobility shift assay (EMSA)

Hek293T cells transiently transfected with the desired constructs were starved for 4 hours in serum-free medium and subsequently stimulated with 5 ng/ml Epo for 15 min or were left untreated. Protein concentrations of the nuclear extracts were measured with the BioRad protein assay. Double-stranded oligonucleotides based on the β-casein promoter (sense: CAGATTTCTAGGAATTC; antisense: GGATTTGAATTCC TAGAAATC) were labelled by filling in 5' protruding ends with Klenow enzyme, using [α-32P]dATP (3,000 Ci/mmol; 10 mCi/ml). This probe binds STAT5 homodimers. Nuclear extracts (5 µg of protein) were incubated with about 10 fmol (20,000 cpm) of probe in gel-shift incubation buffer (10 mM HEPES pH 7.8, 1 mM EDTA, 5 mM MgCl2, 5% glycerol, 5mM DTT, 2 mM pefablock SC, 1 mg/ml BSA, 0.1 mg/ml poly (dI-dC)) for 10 min at room temperature (RT). The supershifting anti-STAT5 antibodies were incubated with the nuclear extracts for 10 min at KT before addition of the radio-labelled β-casein probe. The protein-DNA complexes were separated on

a 4.5%-(w/v)-polyacrylamide gel containing 7.5 % glycerol in 0.5-fold TBE at 20V/cm for 90 min. Gels were fixed in water/methanol/acetic acid (80:10:10, by vol.) for 30 min, dried and autoradiographed.

TAP2 purification and mass spectrometry

Hek293T cells were transfected with the appropriate TAP2 constructs. The TAP2 purification procedure was followed as described previously [17]. Proteins were visualised on a polyacrylamide gel by silver staining, or for mass-spectrometry analysis with Sypro Ruby protein gel stain according to the manufacturers' instructions (Molecular probes). Proteins of interest were excised, prepared for mass spectrometry and applied for nano-LC-MS/MS analysis on an Ultimate (Dionex, Amsterdam, The Netherlands) in-line connected to an Esquire HCT ion trap (Bruker Daltonics, Bremen, Germany).

Modeling method

Molecular models were built for the CIS-elonginB-elonginC complex, using the crystal structure of the SOCS2-elonginB-elonginC complex as a template [38]. The sequences of CIS and SOCS2 were automatically aligned using the sequence alignment editor of moe (chemical computing group). Using this alignment, 150 models were built for the CIS-elonginB-elonginC complex in MODELLER version 8.1 [39], 10 models with the best DOPE and molpdf scores were selected, and evaluated.

Results

Design of MAPPIT experiments

We previously reported a new two-hybrid method to study protein-protein interactions in intact mammalian cells, termed MAPPIT (MAmmalian Protein-Protein Interaction Trap) [36]. Briefly, a bait protein is C-terminally linked to a chimeric EpoR/LR that is deficient in STAT3 recruitment, whilst a prey protein is attached to the string of 4 functional STAT3 recruitment sites of gp130. Association of bait and prey leads to STAT3 activation and subsequent activation of the STAT3-responsive rat Pancreatitis Associated Protein I (rPAPI) promoter-luciferase reporter. To examine

interactions with the EpoR, we used its intracellular receptor tyrosine motifs as baits. We also analysed interactions with the LR itself by mutating the STAT3 recruiting Y1138 to phenylalanine. One or both of the two membrane-proximal tyrosines at positions Y985 and Y1077 were mutated to phenylalanine to examine tyrosine-specific interactions. These LR mutants were termed LR(YFF) and LR(FYF). MAPPIT configurations used in this manuscript are shown in Figure 1A.

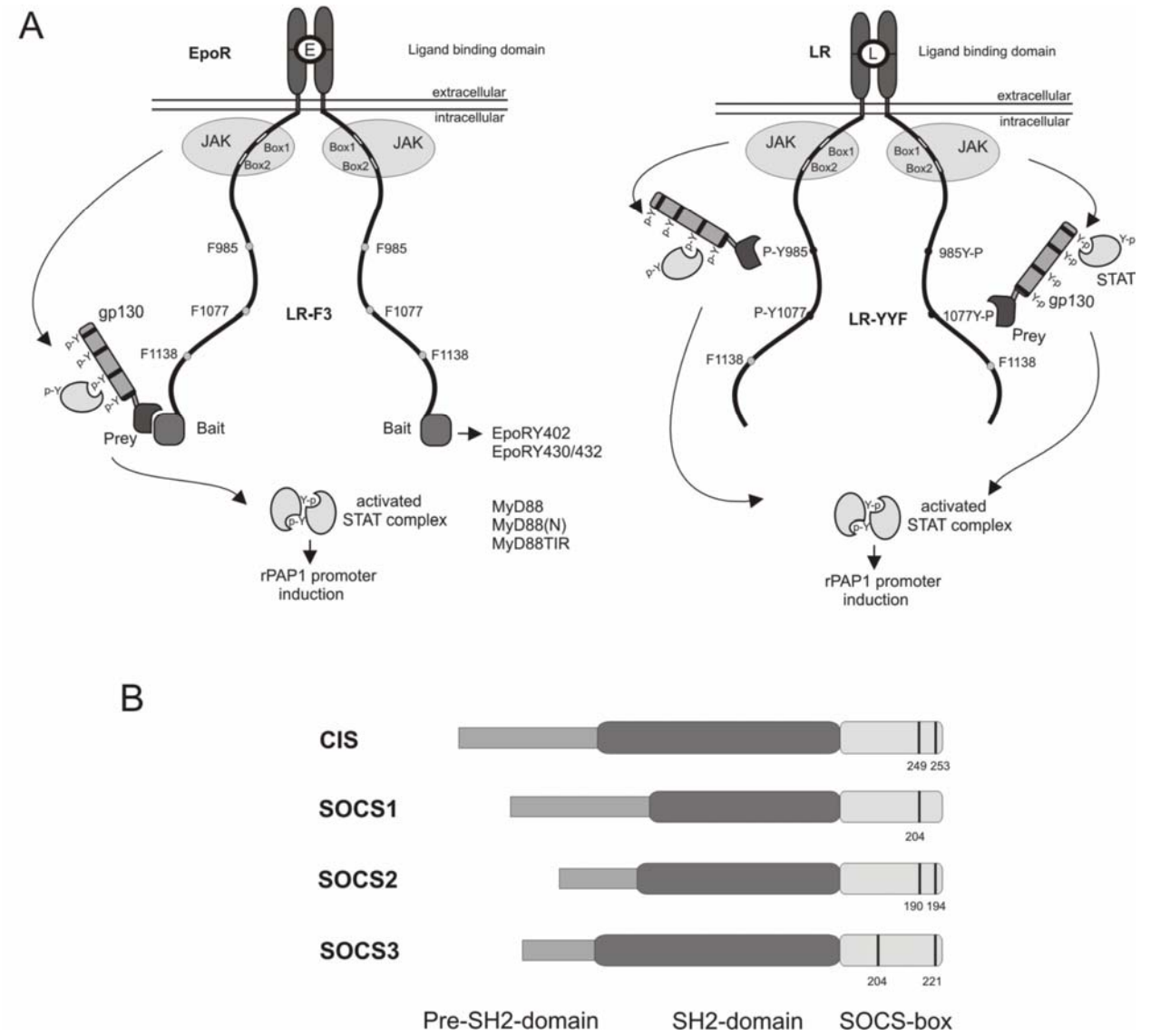

Figure 1: MAPPIT.
A. MAPPIT, a cytokine receptor-based two-hybrid method, is displayed in the left panel, with the various receptor motifs used in this study. The right panel shows a variant of the MAPPIT technique using the STAT3 signalling-deficient LR as bait. Both MAPPIT methods are described in more detail in the results section.
B, Schematic structure of SOCS proteins. Conserved tyrosines in the SOCS-box are indicated with a black box.

The C-terminus of CIS but not of SOCS2 is required for receptor binding

We recently showed interaction of CIS and SOCS2 with the EpoR and the LR. CIS and SOCS2 both interact with Y402 and to a lesser extent also with Y344 and the double Y430/Y432 motifs of the EpoR, and with the Y1077 position in the LR. Although both are highly related, only CIS associated with Y985 of the LR and only SOCS2 with the pY480 motif of the EpoR [17,35]. Here we examine the binding modus of CIS and SOCS2 in more detail. It is well established that interaction of SOCS proteins with their receptor targets depends on their SH2 domains [40,41]. Figure 1B shows a diagrammatic presentation of the structure of SOCS proteins. For MAPPIT use, CIS and SOCS2 preys were generated by N-terminal fusion with part of gp130. Figure 2A shows the critical requirement of the CIS SOCSbox for CISprey binding to the EpoRY402 motif in a MAPPIT experiment. C-terminal deletion of the entire SOCS-box resulted in complete loss of MAPPIT signalling. Detailed mapping showed that deletion of the 5 C-terminal aa and even a single Y253F mutation leads to impaired MAPPIT signalling (Figure 2A). In contrast, no effect was observed upon replacing the second conserved tyrosine at position 249 with phenylalanine. We next performed a similar analysis for SOCS2. Here, deletion of the entire SOCS-box, or Y to F mutation of both conserved tyrosines did not significantly affect signalling (Figure 2B). Very similar observations were obtained in MAPPIT experiments for the EpoR Y430/Y432 motif (Figure 2C). In figure 2D we further expand this dataset to the LR Y985 and Y1077 positions, demonstrating that these findings are not limited to the EpoR system.

CIS binding to the EpoR pY402 motif was also evaluated by phosphopeptide affinity chromatography. Figure 2E clearly shows loss of CIS binding by deletion of the SOCSbox or by introduction of the single Y253F mutation. In contrast to the MAPPIT dataset, complete loss of binding is also observed with the Y249F mutant. This may be explained by a lowered binding affinity so that the interaction with the EpoR pY402 motif is still detected with MAPPIT but not with peptide affinity chromatography. In line with such assumption, MAPPIT detects interactions without the need for any purification step.

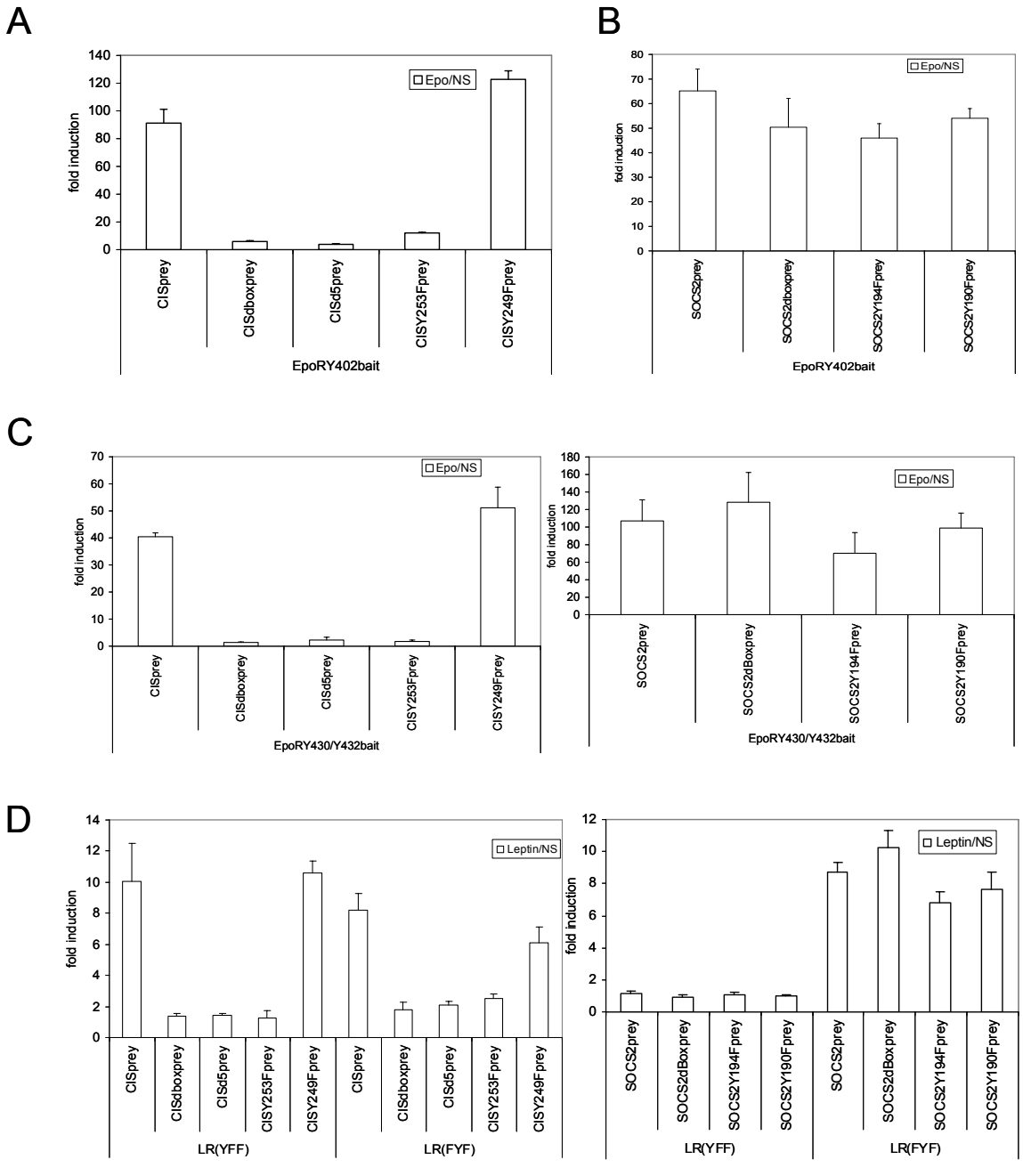
A
fold induction
Epo/NS
CISprey
CISdboxprey
CISd5prey
CISY253Fprey
CISY249Fprey
EpoRY402bait
B
fold induction
Epo/NS
SOCS2prey
SOCS2dboxprey
SOCS2Y194Fprey
SOCS2Y190Fprey
EpoRY402bait
C
fold induction
Epo/NS
CISprey
CISdboxprey
CISd5prey
CISY253Fprey
CISY249Fprey
EpoRY430/Y432bait
fold induction
Epo/NS
SOCS2prey
SOCS2dBoxprey
SOCS2Y194Fprey
SOCS2Y190Fprey
EpoRY430/Y432bait
D
fold induction
Leptin/NS
CISprey
CISdboxprey
CISd5prey
CISY253Fprey
CISY249Fprey
LR(YFF)
LR(FYF)
fold induction
Leptin/NS
SOCS2prey
SOCS2dBoxprey
SOCS2Y194Fprey
SOCS2Y190Fprey
LR(YFF)
LR(FYF)
E
CL
Y402
pY402
fSOCS2
fCIS
fCIS Y249F
fCIS Y253F
fCISdbox

Figure 2: The CIS C-terminus is critical for interaction with EpoR motifs.
A. Interaction of CISprey mutants with EpoRY402. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of the pMG2-CIS prey construct and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. All preys were also tested for interaction with a "mock bait" lacking an EpoR tyrosine motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS and s.d. of triplicate measurements are plotted.
B. Interaction of SOCS2prey mutants with EpoRY402. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of the pMG2-SOCS2 prey construct and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. All preys were also tested for interaction with a "mock bait" lacking the EpoR motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS + s.d.
C. Interaction of CIS/SOCS2prey mutants with EpoRY430/Y432. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY430/Y432 bait, various mutants of the pMG2-CIS and pMG2-SOCS2 prey constructs and with the pXP2d2-rPAP1-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. All preys were also tested for interaction with a "mock bait" lacking the EpoR motif, and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS+ s.d.
D. Interaction of CIS/SOCS2prey mutants with the LR(YFF) and LR(FYF). Hek293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants, various mutants of the pMG2-CIS or pMG2-SOCS2 prey constructs and with the pXP2d2-rPAP1-luci reporter. The transfected cells were either stimulated for 24 hours with leptin or were left untreated (NS). Luciferase measurements were performed in triplicate. All preys were also tested for interaction with the LR lacking intracellular tyrosines and consistently showed absence of any signaling (data not shown). Data are expressed as ratio stimulated/NS+ s.d.
E. Peptide affinity chromatography. Hek293T cells were transfected with various mutants of CIS or SOCS2. The lysates were incubated with the (phospho-)tyrosine peptides corresponding to the Y402 motif of the EpoR. Specific protein binding was revealed by SDS-PAGE and immunoblotting using the anti-FLAG antibody.

No role for the SOCS1 and SOCS3 SOCS-box for receptor binding

We also examined the role of the SOCS-box for the inhibitory function of SOCS1 and SOCS3. In this assay system, with clear inhibition of EpoR and LR signal transduction by co-expression of SOCS1 and SOCS3, deletion of the complete SOCS-box of SOCS1 or SOCS3 did not significantly alter the inhibitory effect (Figure 3A-B). We conclude that the SOCS-box of SOCS1 and 3, in analogy with SOCS2, does not contain critical determinants involved in substrate binding. This highlights the unique new property of the CIS SOCS-box, which we next evaluated in more detail.

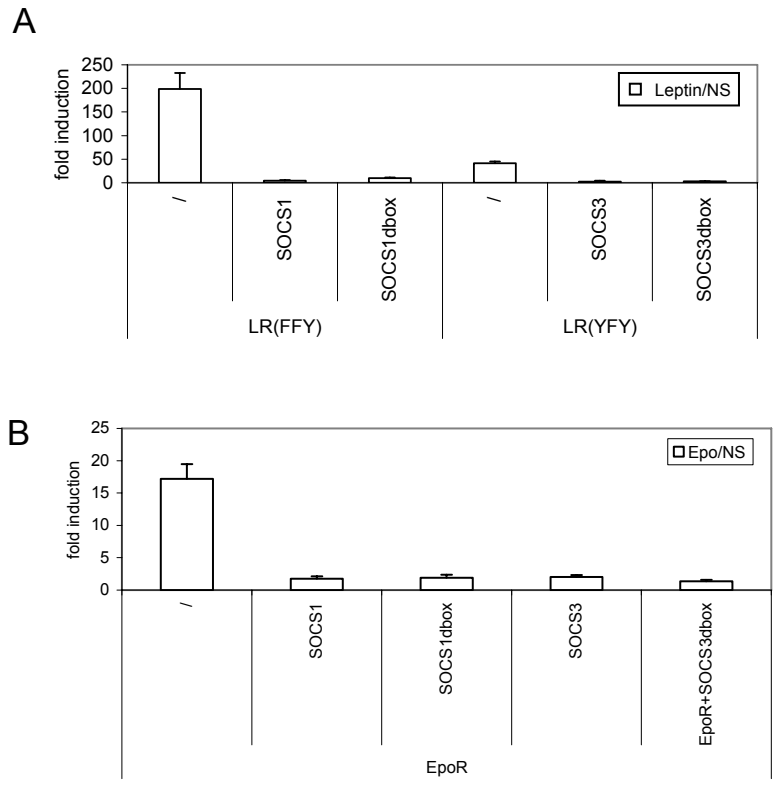

Figure 3: The C-terminus of SOCS1 and SOCS3 is not essential for signalling inhibition.
A-B. Inhibition of EpoR and LR signalling by SOCS1/SOCS3 mutants. Hek293T cells were transiently co-transfected with plasmids encoding different LR tyrosine mutants or EpoR, various mutants of SOCS1 and SOCS3 and with the pXP2d2-rPAP1-luci reporter. The LR tyrosine mutants are used to minimize interference of other inhibitors. The transfected cells were either stimulated for 24 h with ligand or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS+ s.d.

Critical role of Y253 in CIS function

We first investigated the functional implications of the CIS SOCS-box mutations. CIS operates in a classical negative feedback loop on EpoR signalling: it is rapidly and strongly induced by activated STAT5 upon EpoR activation, subsequently binds to the Y402 site in the EpoR, and there inhibits STAT5 activation. We first looked at the effect of CIS mutations on STAT5-dependent β-casein reporter activity using the EpoR Y402 bait. Wild-type CIS clearly abrogated reporter induction as expected. In contrast, co-expression of mutant proteins with C-terminal deletion of the SOCS-box, or with the Y253F mutation was unable to impair reporter induction. The CIS Y249F mutant inhibited signalling to a similar extent as wild type CIS protein (Figure 4A). Expression of all CIS variants was verified via Western blot (not shown).

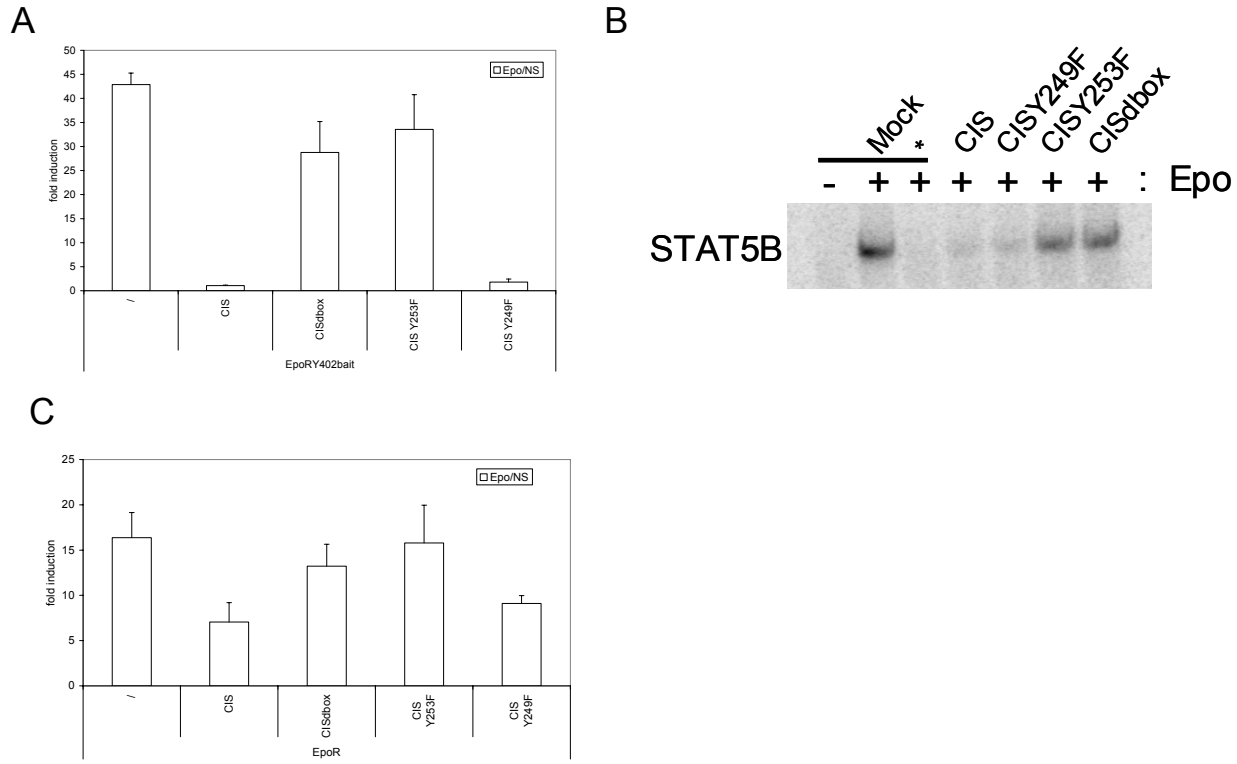

Figure 4: Critical role of CIS C-terminus in blocking STAT5 activation.
A. STAT5 reporter assay using the EpoRY402 motif. Hek293T cells were transiently co-transfected with plasmids encoding the EpoRY402 bait, various mutants of FLAG-tagged CIS and with the pGL3-5xbeta-casein-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. Data are expressed as ratio stimulated/NS + s.d.
B, EMSA using the EpoRY402 motif. Hek293 Flp-In cells were transiently co-transfected with plasmids encoding the EpoR Y402 bait, various FLAG-tagged CIS mutants and STAT5B. Nuclear lysates were incubated with ^{32}P labelled probe corresponding to a β-casein STAT5 binding site to reveal active STAT5 complexes.
C, STAT5 reporter assay using the WT-EpoR. Hek293T cells were transiently co-transfected with plasmids encoding the EpoR, various mutants of FLAG-tagged CIS and with the pGL3-5xbeta-casein-luci reporter. After transfection, cells were left untreated (NS) or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. Data are expressed as ratio stimulated/NS + s.d.

Confirmation was obtained using electrophoretic mobility shift assays. A ^{32}P labelled probe corresponding to a β -casein STAT5 binding site was used to visualise bound STAT5 complexes. Whilst wild-type CIS clearly suppressed the formation of nuclear STAT5-DNA complexes, deletion of the complete SOCS-box, as well as the Y253F mutant, resulted in loss of inhibition (figure 4B). Again, the Y249F mutant behaved as wild-type CIS. Supershift with anti-FLAG antibody confirmed the presence of STAT5B in the complexes (not shown).

Reporter assays were also performed on the wild-type EpoR. Although the effects were less pronounced, the tendencies clearly corresponded to what we observed for

the EpoR bait construct (figure 4C). This weaker effect is most likely explained by the incomplete overlap of STAT5 and CIS binding sites [6,35].

CIS interaction with MyD88 does not depend on its SOCS-box

SOCS proteins are also rapidly induced after Toll-like receptor stimulation. However, no interaction partner of the SOCS proteins in TLR signalling has been described so far. A possible target of SOCS proteins in TLR signalling is the adaptor protein MyD88, which is used by most TLRs. To investigate this possibility, we performed MAPPIT analysis using MyD88 as bait and the CISprey. As shown in Figure 5A, we observed clear induction of luciferase activity implying interaction between CIS and MyD88. To investigate the role of the SOCS-box of CIS in this interaction, we next analysed the effect of the abovementioned CIS mutants. Much in contrast to association of CIS with the EpoR and LR, interaction between MyD88 and CIS was not affected by any CIS mutation, including deletion of its entire SOCS-box. These data were confirmed by co-immunoprecipitation (figure 5B). Here, we transiently co-expressed E-tagged MyD88 (MyD88-E) and FLAG-tagged CIS (CIS-FLAG), CIS Y253F (CISY253F-FLAG) or CIS lacking its SOCS-box (CISdBox-FLAG). In every case, MyD88-E was co-immunoprecipitated using an anti-FLAG antibody.

MyD88 consists of two interaction domains: a C-terminal 'TIR' domain and a N-terminal 'Death Domain', linked together by a short intermediate domain. To examine the role of either domain in CIS binding, we created MAPPIT baits containing the N-terminal part of MyD88 encompassing the Death Domain and the intermediate domain, or the C-terminal TIR domain. MAPPIT analysis clearly shows that only the N-terminal part of MyD88 interacts with CIS (figure 5C). Again, co-immunoprecipitation studies confirmed these findings (figure 5D). Together, we here clearly document a role for the MyD88 Death Domain in CIS recruitment, and show that this interaction solely depends on the CIS SH2 domain.

C-terminal mutations in CIS do not affect Elongin B/C and cullin5 recruitment

We used a variant of the Tandem Affinity Purification (TAP) method developed by Puig and co-workers [42] (S.E., unpublished results) to analyse protein complexes of CIS and its mutants. Cullin5 and Elongins B and C were identified as interacting

partners of CIS. While deletion of the complete SOCS-box of CIS abrogates their association, mutation of the tyrosines at position 249 or 253 to phenylalanine did not significantly influence Cullin5 or Elongin B and C binding (Figure 6A).

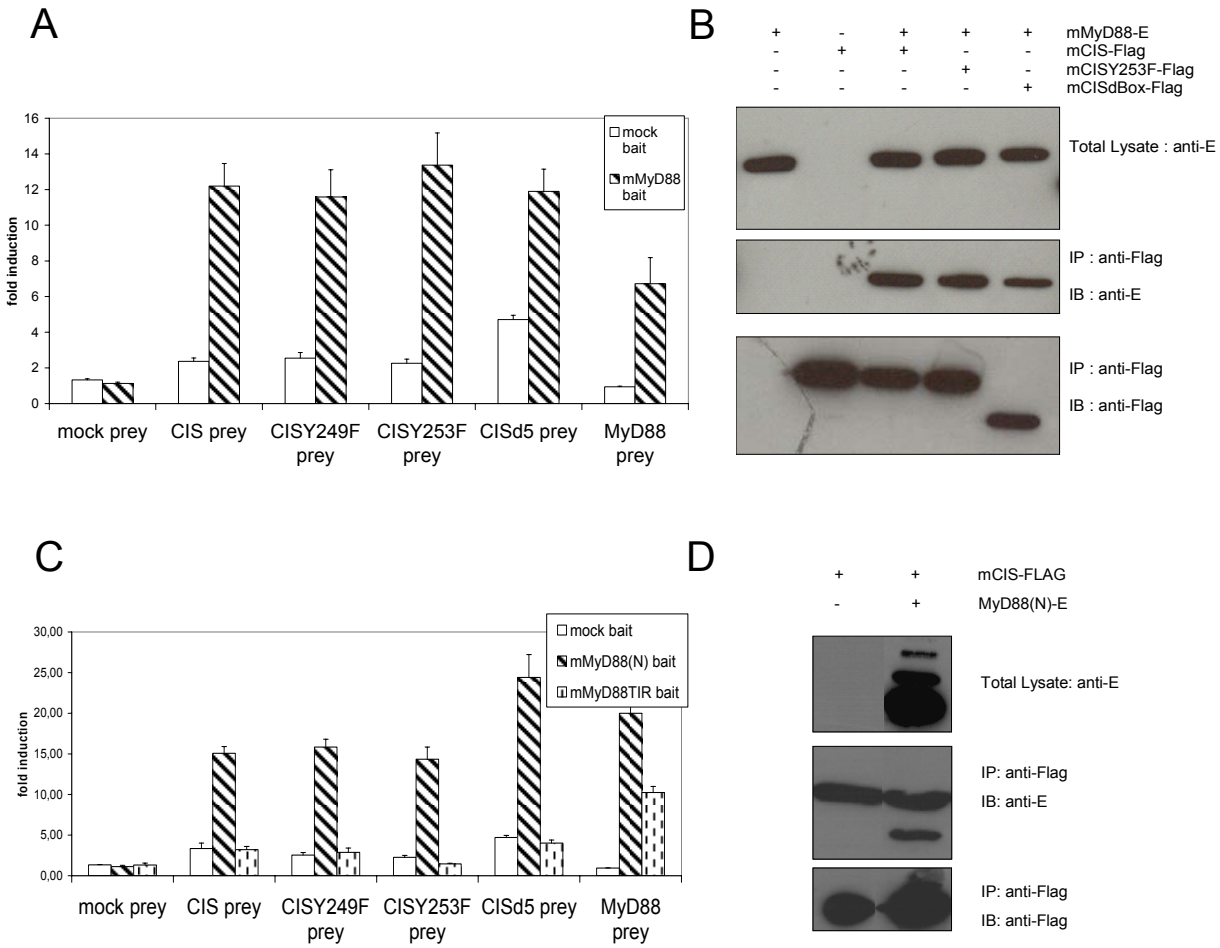

Fig. 5. The SOCS-box of CIS is not critical for interaction with MyD88.
A, Interaction of MyD88 and CIS prey constructs. Hek293T cells were transiently co-transfected with the MAPPIT mock bait or the mMyD88 bait plasmid (0.1 µg), various CIS or CIS-mutant prey (0.5 µg) constructs and with the pXP2d2-rPAP1-luci reporter (0.2 µg). MyD88 prey was used as a positive control. The transfected cells were either stimulated for 24 h with Epo or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS + s.d.
B, Co-immunoprecipitation analysis. Hek293T cells were transiently co-transfected with combinations of mMyD88-E, mCIS-FLAG, mCISY253F and mCISdBox. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E.
C, Role for the N-terminal domain of MyD88 in CIS binding. Hek293T cells were transiently co-transfected with the MAPPIT mock bait, the mMyD88(N) bait or the mMyD88TIR bait vectors (0.1 µg), various CIS or CIS-mutant prey (0.5 µg) constructs and with the pXP2d2-rPAP1-luci reporter (0.2 µg). The MyD88 prey construct was used as a positive control. The transfected cells were either stimulated for 24 h with Epo or were left untreated (NS). Luciferase measurements were performed in triplicate. Data are expressed as ratio stimulated/NS + s.d.
D. Co-immunoprecipitation analysis. Hek293T cells were transiently co-transfected with combinations of mMyD88(N)-E, and mCIS-FLAG. Cell lysates were immunoprecipitated (IP) with anti-FLAG and subsequently immunoblotted (IB) with anti-E.

Modeling of the SOCS-box of CIS

We used the crystal structure of the SOCS2-elongin C-elongin B complex [38] to build a molecular model for CIS, bound to Elongin B and C (Figure 6C). This model, together with sequence alignment with other SOCS proteins (Figure 6B) showed that residues involved in Elongin C binding are very conserved. As for SOCS2, the C-terminal residues of CIS, mutated in this work are not part of its actual Elongin C-binding site and mutation of the C-terminus of CIS is thus not predicted to directly affect Elongin C binding. As in SOCS2, the C-terminus of CIS is buried in the interface between the SH2 domain and the SOCS-box domain. The hydroxyl group of the completely buried Y253 hydrogen bonds to the buried C-terminal carboxyl group. The C-terminus of CIS or SOCS2 is not able to make direct contact with a phosphopeptide substrate bound to the SH2 domain (Figure 6C).

Discussion

SOCS proteins typically consist of a phosphotyrosine binding SH2 domain, a C-terminal SOCS-box involved in proteasome recruitment and a pre-SH2 domain that only in the case of SOCS1 and SOCS3 contains a JAK kinase blocking KIR domain. Association of SOCS proteins with their target substrates is believed to occur solely via their SH2 domain. We here took a closer look at the binding modus of SOCS proteins using the MAPPIT approach, combined with biochemical and functional analyses.

A key finding is that the SOCS-box of CIS is essential for association with recruitment motifs in cytokine receptors including the EpoR and LR. Deletion of the entire SOCS-box completely abrogated binding, and more detailed mutagenesis analysis revealed the critical role of the single C-terminal Y253 residue. These findings were confirmed by peptide affinity chromatography using the phosphorylated or non-phosphorylated EpoRY402 motif. Furthermore, reporter assays and EMSA experiments extended these findings to functional activation of STAT5. Indirect effects of the mutations on the structural integrity of CIS could be ruled out since clear SOCS-box-independent interaction was observed with the unrelated MyD88 protein as bait. Association of the Y249F CIS mutant with the EpoR $_{P}$Y402 motif could not be demonstrated by peptide affinity chromatography, suggesting Y249 might serve a similar role as Y253.

However, MAPPIT experiments showed indisputable association of this CIS mutant with the same EpoRY402 motif. Only this latter interaction was functionally confirmed by the clear inhibitory effect seen in EpoRY402-dependent STAT5 recruitment and activation. The Y249F mutant thus only modestly reduced binding affinity compared to wild-type CIS. This reduced binding affinity of CISY249F completely abolished binding with the EpoR pY402 motif in a peptide affinity chromatography experiment, much in contrast with MAPPIT. The MAPPIT technique therefore reveals itself as a sensitive tool for the identification of weaker, but functionally highly relevant protein interactions.

In line with our findings that the SOCS-box of CIS is essential for EpoR association, Ketteler et al. previously reported that the SOCS-box of CIS is essential for the apoptotic effect of CIS on erythroid progenitor cells. Seemingly contradictory to our observations they also found that the SOCS-box of CIS was not required for inhibition of EpoR-induced proliferative responses [10]. However, this anti-proliferative effect may be due to CIS interference with intermediate signalling molecules coupling to the cell cycle. CIS can indeed associate with downstream effector molecules in a SOCS-box independent modus as we showed for MyD88.

This critical role of the CIS SOCS-box in substrate binding may be a unique feature of CIS, and was not seen for the highly related SOCS2 protein, or for SOCS1 and -3. The corresponding mutation of the conserved tyrosine in the SOCS-box of SOCS2 or even deletion of its entire SOCS-box did not show any significant effect on receptor association. Likewise, the inhibitory effect of SOCS1 and SOCS3 on cytokine receptor signalling was hardly affected by removal of the SOCS-box. Previously, the SOCS-box of SOCS1 was also reported dispensable for LIF, IL-6 and GHR signalling inhibition but not for G-CSF signal transduction [40,41,43,44]. In vivo deletion of the entire SOCS-box of SOCS1 however leads to partial loss of SOCS1 function [45], most likely reflecting its role in Elongin B/C binding, thus establishing an E3 ubiquitin ligation complex leading to proteasomal degradation of associated receptor complexes.

Using MAPPIT, we could also demonstrate the association of CIS with the universal TLR adaptor MyD88. This interaction was confirmed by co-immunoprecipitation. In contrast to the data described above, mutation of the conserved C-terminal tyrosine at position 253 to phenylalanine or deletion of the complete SOCS-box of CIS had no effect at all on MyD88 binding. Further analysis of this association revealed a critical

role for the Death Domain of MyD88 in CIS binding. More studies are required to elucidate the functional consequences of this interaction. We also observed interaction of other members of the SOCS protein family with MyD88 and its splicing variant lacking the intermediate domain (P.U., unpublished results), and structural and functional analyses of these interactions are ongoing. Interestingly, our results imply differential modulation by CIS of signalling via cytokine receptors and Toll-like receptors.

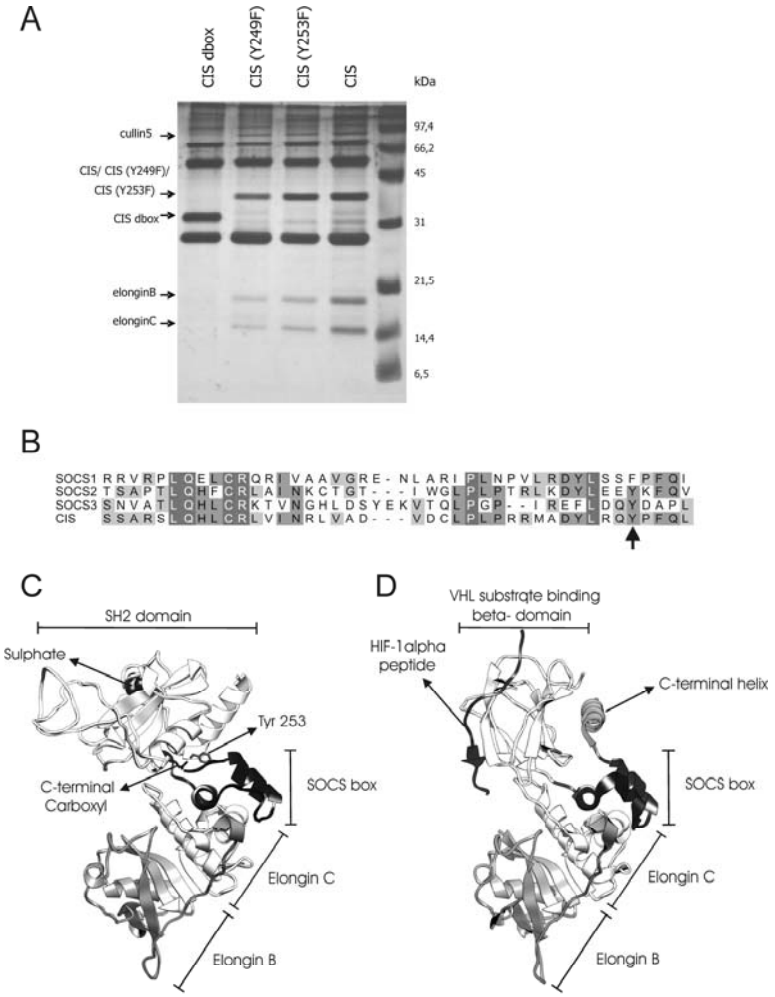

Figure 6: Role of the CIS C-terminus in ElonginB/C association.
A, TAP2 purification using CIS mutants. Hek293T cells were transiently transfected with various mutants of the pMet7TAP2-CIS construct. Cell lysates were purified by the TAP2 method and were then loaded on 14% polyacrylamide gel. After silver-staining the protein bands indicated with an arrow were analysed by mass spectrometry and identified as the annotated protein.
B. Sequence alignment of SOCS-boxes. SOCS-box sequences of murine SOCS1,2 and 3 and CIS were aligned using the t_coffee algorithm. An arrow indicates the Y253 positions. Increasing grey shading corresponds with increasing % identity.
C. Homology model of mouse CIS, in complex with Elongin C and B. The CIS SOCS-box is shown in black. The position of the phosphopeptide substrate in the model is indicated by a sulphate ion, copied from the SOCS2 template structure, that mimics the phosphate group of the phosphopeptide substrate. Y253 hydrogen bonds with the C-terminal carboxyl group.
D. Crystal structure of VHL protein (1LQB), bound to its hydroxylated HIF-1α substrate (black), and to Elongins C and B. The pVHL SOCS-box and the hydroxyproline binding domain are in black. The extra C-terminal helix (dark grey) is indicated.

A crystal structure for SOCS2 in a complex with elongin C and B was recently determined [38]. A molecular model was built for the CIS-elonginC-elonginB complex in order to get structural insight into the role of the CIS C-terminus on substrate recognition. In the SOCS2 structure and the CIS model, the C-terminus is buried in the interface between the SH2 domain and the SOCS-box excluding the possibility that this C-terminus could make direct contact with a phosphopeptide substrate bound to the SH2 domain (figure 6C). In both CIS and SOCS2, the hydroxyl group of the last tyrosine hydrogen bonds to the buried C-terminal carboxyl group. The Y253F mutation in CIS can thus be expected to influence the protein structure or folding: removing the tyrosine hydroxyl group may render burial of the C-terminus energetically unfavorable. The tyrosine at position 253 of CIS may therefore play a structural role. One can speculate that this affects stability of the packing between SOCS-box and SH2 domain, but how this might affect binding to a phosphopeptide substrate remains unclear. Allosteric effects on the substrate binding pocket cannot be excluded. However, the direct environment of the C-termini in the CIS model and SOCS structure are very similar, hinting that the same phenomenon would be expected for the Y194F mutation in SOCS2, while this mutation has no effect on interaction with its phosphopeptide substrates.

Tyrosine phosphorylation of SOCS proteins has been reported before. Cacalano et al. showed Epo-induced phosphorylation of the two conserved tyrosines in the SOCS-box of SOCS3, including Y221 that corresponds to the Y253 position in CIS. Interestingly, the C-terminal $_{P}$Y221 allowed binding and functional coupling to the Ras signalling pathway on the one hand while both phosphorylated tyrosines, situated centrally and C-terminally in the SOCS-box, were involved in abrogation of Elongin C interaction [46,47]. Intriguingly, as in CIS and SOCS2, Y221 in SOCS3 is also predicted (not shown) to be buried in the interface between the SH2-domain and the SOCS-box, and its hydroxyl group hydrogen bonds to the buried C-terminal carboxyl group. It is therefore likely that phosphorylation of Y221 in SOCS3, and possibly Y253 in CIS, requires changes in the conformation of the C-terminus. One possibility is that burial of the C-terminus as seen in the SOCS2 crystal structure depends on binding of the Elongin complex. In the absence of Elongin binding, the C-terminal tyrosine motifs may be accessible for phosphorylation. In this structural modus, phosphorylation-dependent interactions may occur with signaling molecules

or with accessory proteins that facilitate interactions with (a subset of) substrates. Mutating Y253 in CIS may then prevent the phosphorylation-driven structural change required for downstream interactions. Deletion of the 4 C-terminal aa in the CISprey, eliminating the putative phosphorylation context, leads to impaired MAPPIT signaling, adding evidence to this phosphorylation hypothesis (not shown). Of note, it seems unlikely that disturbed Elongin binding causes the substrate binding defects in the CIS mutants: TAP2 purification of wild type CIS and of its C-terminal tyrosine mutants showed clear association with Elongins B and C and with Cullin 5, which was completely lost when the entire SOCS-box of CIS was deleted. Thus, Elongin C binding does not involve the C-terminus of CIS and has no role in the effect of mutating Y253.

The role of the C-terminus of CIS in substrate binding is remarkably similar to the role of the C-terminal helix of the Von Hippel-Lindau (VHL) tumour suppressor protein. The VHL protein is part of a VHL/E3 ligase complex involved in ubiquitination of transcription factors like hypoxia inducible factor (HIF)-1α, targeting them for degradation. The VHL/E3 ligase complex binds and ubiquitinylates two oxygen-dependent degradation domains (HIF-ODDD). The crystal structure of VHL protein, bound to a hydroxylated HIF-1 alpha peptide, and to the Elongins C and B has been determined (figure 6D) [48]. This revealed that the SOCS-box of VHL is followed by a C-terminal helix which is not directly involved in binding to the hydroxylated HIF-1□peptide. Like the C-terminus of CIS, this helix tightly interacts with the SH2 domain. Lewis et al. recently reported that this C-terminal helix is critical for ubiquitination of HIF-1α. Deletion of this C-terminal peptide impaired VHL binding and ubiquitinylation of the C-terminal HIF-ODDD, while ubiquitinylation and degradation of the N-terminal HIF-ODDD is hardly affected [49]. The role of the C-terminal peptide in VHL thus shows some striking parallels with the CIS C-terminus. As for CIS, deletion of the VHL C-terminus specifically affects certain functions/interactions, leaving other functions unaltered. It was suggested that deletion of the C-terminus might affect VHL substrate binding by secondary folding effects.

In brief, we have shown that the SOCS-box of CIS is essential for interaction with target cytokine receptors but not with the universal TLR adaptor MyD88. It appears that the biological role of the SOCS-box is more complex than simple recruitment of

a ubiquitin-ligation complex, and is also involved in (regulated) substrate binding. Depending on the type of SOCS protein, this may include receptor recruitment motifs, alternative signalling pathways, and other SOCS proteins [16,17]. The precise underlying controls that are involved in these diverse functions of the SOCS-box remain to be clarified.

Acknowledgements

We greatly acknowledge following colleagues for sharing research tools: Robyn Starr (CIS,SOCS1 and SOCS2 constructs), Ivo Touw (pGL3-β-casein-luciferase reporter), Walter Becker (pECE-STAT5B expression vector) and Rudy Beyaert (pCAGGSE-mMyD88 expression vector). We also thank Marc Goethals for peptide synthesis and An Staes and Evy Timmerman for mass spectrometry analysis. This work was supported by grants from the Flanders Institute of Science and Technology (GBOU 010090 grant, and to P.U.), from The Fund for Scientific Research – Flanders (FWO-V Grant N° 1.5.446.98, and to D.L. and S.E.), from Ghent University (GOA 12051401)

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Chapter 10: Functional cross-modulation between SOCS proteins can stimulate cytokine signalling.

Both overexpression and deficiency of SOCS2 causes a remarkable gigantism phenotype in mice (Greenhalgh et al., 2002a; Greenhalgh et al., 2002b; Metcalf et al., 2000). In vitro data also demonstrate a dual effect of SOCS2 on GH signalling with low SOCS2 concentrations having an inhibitory effect while signalling is restored at higher concentrations (Favre et al., 1999). In this article we studied the mechanism underlying this observation. We showed that SOCS2 can interfere with the inhibitory function of SOCS1 and SOCS3 in leptin, GH and type I IFN signalling. In analogy to SOCS2 interference with CIS interaction at the LR as described in chapter 8, this regulatory capacity of SOCS2 depended on elongin B/C recruitment to its SOCS-box. We observed degradation of SOCS1 by SOCS2 but not by the elongin B/C recruitment-deficient mutant suggesting that SOCS2 targets other SOCS proteins for proteasomal degradation. By means of MAPPIT experiments we demonstrated that SOCS2 can associate with all members of the SOCS protein family. This phenomenon was also observed for SOCS6 and SOCS7. Comparing SOCS6 with an elongin B/C deficient SOCS6 mutant generated similar data in degradation experiments and reporter assays, suggesting that this protein may also negatively regulate SOCS activity.

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FUNCTIONAL CROSS-MODULATION BETWEEN SOCS PROTEINS CAN STIMULATE CYTOKINE SIGNALLING.

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In press, Journal of Biological Chemistry

Summary

Suppressors of cytokine signalling (SOCS) proteins are negative regulators of cytokine signalling that function primarily at the receptor level. Remarkably, in vitro and in vivo observations revealed both inhibitory and stimulatory effects of SOCS2 on growth hormone (GH) signalling suggesting an additional regulatory level. In this study, we examined the possibility of direct cross-modulation between SOCS proteins and found that SOCS2 could interfere with the inhibitory actions of other SOCS proteins in GH, interferon (IFN) and leptin signalling. This SOCS2 effect was SOCS-box dependent, required recruitment of the elongin BC complex and coincided with degradation of target SOCS proteins. Detailed MAPPIT analysis indicated that SOCS2 can interact with all members of the SOCS family. SOCS2 thus may function as a molecular bridge between an E3 ubiquitin ligase complex and SOCS proteins targeting these for proteasomal turnover. We furthermore extended these observations to SOCS6 and SOCS7. Our findings point to a unique regulatory role for SOCS2, -6 and -7 within the SOCS family and provide an explanation for the unexpected phenotypes observed in SOCS2 and SOCS6 transgenic mice.

Introduction

Cytokine signalling typically is a transient event, implying rapid and finely tuned attenuation. Receptor binding leads to rapid activation of receptor-associated members of the Janus family of kinases (JAKs). Subsequent phosphorylation of tyrosine residues in the receptor tails enables recruitment of downstream signalling molecules whereby the signal transducers and activators of transcription (STATs) play a prominent role. Activated STATs translocate to the nucleus where they control

cytokine-regulated gene transcription. Negative control occurs at many levels and involves receptor down-modulation, protein tyrosine phosphatases, protein inhibitors of activated STATs (PIAS) and members of the SOCS protein family.

The cytokine-inducible suppressors of cytokine signalling (SOCS) family consists of eight different members (SOCS1-7 and CIS) characterized by conserved structural features. All SOCS proteins consist of a central SH2 domain flanked by a variable N-terminal region and a conserved C-terminal SOCS-box (1,2). The SH2-domain can inhibit STAT activation by direct competition for the phosphorylated receptor recruitment sites (3-8). SOCS1 and SOCS3 carry an additional kinase inhibitory region (KIR) domain in their N-terminal region, which acts as a pseudosubstrate for the JAK kinase thereby blocking signalling (5). The SOCS-box was shown to act as an interaction domain for the elongin BC complex (9,10) that, in turn, is a component of an E3 ubiquitin ligase complex (11). This way, the SOCS-box can control protein turn-over by marking target proteins for proteasomal degradation (12). However, the significance of the interaction between SOCS proteins and the elongin BC complex is not totally clarified as some reports propose that elongin association targets SOCS molecules for proteasomal degradation (10,12-15), while other data suggest that elongin BC binding stabilise SOCS protein expression (9,16,17).

SOCS gene deletion studies in mice have underscored their importance in specific, restricted cytokine signalling pathways. E.g., SOCS1-deficient mice suffer from deregulated interferon-γ (IFN-γ) signalling characterised by malfunctioning of the immune system at several levels (18-20) and SOCS3 haplo-insufficient mice or mice with specific deletion of SOCS3 in hypothalamic neurons show augmented central leptin sensitivity (21,22), suggesting a key role for SOCS3 in leptin resistance. A special case concerns SOCS2 that can have opposing effects on growth hormone (GH) signalling: SOCS2 knock-out mice exhibit an overgrowth phenotype due to prolonged GH-dependent STAT5 activity (23,24) and paradoxically, overexpression of SOCS2 in a transgenic mouse model also leads to gigantism (25). This dual effect of SOCS2 is also observed in vitro, where low SOCS2 doses moderately inhibit GH signalling while higher levels positively regulate signalling (25-27). A similar phenomenon is observed for SOCS2 on prolactin (PRL) (28) and IL-3 signalling (29). The role of SOCS2 in regulating cytokine-induced signals is obviously complex since increasing SOCS2 levels can overcome the negative effect of SOCS1 on GHR and PRL

signalling and can partially restore the SOCS3 downregulated PRL function (26,28,30). Of note, SOCS6 overexpression also confers an enhanced phenotype since 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 -3 that are 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.

The present study was conducted to clarify the stimulatory effect of SOCS2 observed in GH signalling. 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 as well as the elongin BC binding motif in the SOCS2 SOCS-box, supporting proteosomal 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. Starr. The pMET7-mouse LR long form (pMET7-mLR) was a gift from Dr. Tartaglia and the pcb6-rbGHR vector was a gift from Dr. Strous. The mouse thymus cDNA was kindly provided by Dr. Brouckaert. The pMET7-Flag-SOCS3 expression vector was described elsewhere (33). The expression vectors pMET7-Flag-CIS, pMET7-Etag-CIS and pMET7-Flag-SOCS2 have been previously described (34). 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 described elsewhere (35,36).

Name of the construct	Template	Cloning vector	Cloning sites	oligos
pMET7-rbGHR-Flag	pcb6-rbGHR	pMET7	EcoRV-XhoI	5'CCGGATATCACCATGGATCTCTGGCAGCTGC 5'CCGCTCGAGTTACTTATCGTCGTCATCCTTGTAATCTGGCAAG ATTTTGTTCAGTTG
pMET7-Flag-SOCS3 Δbox	pMET7-Flag-SOCS3	pMET7	EcoRI-XbaI	5' GCGAGATCTCAGAATTCGTCACCCACAGCAAGTTTCC 5' CGCTTCTAGATTAGTTGGAGGAGAGAGGTCGG
pMET7-Flag-mSOCS1	pEF-Flag-I/mSOCS1	pMET7	EcoRI-XbaI	5' CCAGCGAATTCATGGCGCGCCAGGACTACAAGGAC 5' GGTCGTCTAGATCAGATCTGGAAGGGGAAGGAAC
pMET7-Flag-mSOCS1 Δbox	pEF-Flag-I/mSOCS1	pMET7	EcoRI-XbaI	5' CCAGCGAATTCATGGCGCGCCAGGACTACAAGGAC 5'GGTCGTCTAGATCAGCGGCGCTGGCGCAGCGGGGCCCCCAAC
pMET7-Etag-mSOCS1	pEF-Flag-I/mSOCS1	pMET7-EtagCIS*	SacII-XbaI	5'CGTCCCGCGGTAGCACGCAACCAGGTGGCAG 5'GGTCGTCTAGATCAGATCTGGAAGGGGAAGGAAC
pMET7-Etag-mSOCS1 Δbox	pEF-Flag-mSOCS1	pMET7	SacII-XbaI	5'CGTCCCGCGGGAACCCTGCGGTGCCTGGAGCCCTC 5'GGTCGTCTAGATCAGCGGCGCTGGCGCAGCGGGGCCCCCAAC
pMET7-Etag-SOCS2	pMET7-Flag-SOCS2	pMET7-Etag-CIS	Not-XbaI	5' CGTCGCGGCCGCGGTAACCCTGCGGTGCCTGGAGCCCTC 5' GCAGGTCTAGATTATACCTGGAATTTATATTC
pMET7-Flag-SOCS2 Δbox	pMET7-Flag-SOCS2	pMET7	NotI-XbaI	5' TGCCTTTACTTCTAGGCCTG 5' GCAGGTCTAGATTATGATGTATACAGAGGTTTG
pMET7-Etag-SOCS2 Δbox	pMET7-Etag-SOCS2	pMET7		
pMET7-Flag-SOCS6	pMG2-mSOCS6	pMET7	EcoRI-KpnI	
pMET7-Flag-SOCS2 (LC-QQ)	pMET7-Flag-SOCS2		mutagenesis	5'GTATACATCAGCACCCACTCAGCAGCATTTCCAACGACTCGCC ATTAAC 5'GTTAATGGCGAGTCGTTGGAAATGCTGCTGAGTGGGTGCTGA TGTATAC
pMET7-Etag-SOCS2 (LC-QQ)	pMET7-Etag-SOCS2		mutagenesis	5'GTATACATCAGCACCCACTCAGCAGCATTTCCAACGACTCGCC ATTAAC 5'GTTAATGGCGAGTCGTTGGAAATGCTGCTGAGTGGGTGCTGA TGTATAC
pMET7-Flag-SOCS2 (LC-PF)	pMET7-Flag-SOCS2		mutagenesis	5'GTATACATCAGCACCCACTCCGCAGCATTTCTTTCGACTCGCC ATTAAC 5'GTTAATGGCGAGTCGAAAGAAATGCTGCGGAGTGGGTGCTGA TGTATAC
pMET7-Etag-SOCS2 (LC-PF)	pMET7-Etag-SOCS2		mutagenesis	5'GTATACATCAGCACCCACTCCGCAGCATTTCTTTCGACTCGCC ATTAAC 5'GTTAATGGCGAGTCGAAAGAAATGCTGCGGAGTGGGTGCTGA TGTATAC
pMET7-Flag-SOCS6 (LC-QQ)	pMET7-Flag-SOCS6		mutagenesis	5'TGCAGGTGCGCTCGCAACAGTACCTGCAACGCTTTGTTATCCG T 5'ACGGATAACAAAGCGTTGCAGGTACTGTTGCGAGCGCACCTG CA
CIS SOCS-box bait **	pEF-Flag-I/mCIS	pCEL	SstI-NotI	5'GCGAGAGCTCCGGATCCGCCCGCAGCTTACAACATC 5'CGCTGCGGCCGCTTAGAGTTGGAAGGGGTACTG
SOCS1 SOCS-box bait	pEF-Flag-I/mSOCS1	pCEL	SstI-NotI	5'GCGAGAGCTCAGTCCGGCCGCTGCAGGAGC 5'GCTTGCGGCCGCTTAGATCTGGAAGGGGAAGGA
SOCS2 SOCS-box bait	pEF-Flag-I/mSOCS2	pCEL	SstI-NotI	5'GCGAGAGCTCCGCACCATCTCTGCAGCATC 5'GCTGCGGCCGCTTATACCTGGAATTTATATTCTTCC
SOCS3 SOCS-box bait	pMET7-Flag-SOCS3	pCEL	SstI-NotI	5'CGAGAGCTCCGTGGCTACCCTCCAGCATC 5'CGCTGCGGCCGCTTAAAGTGGAGCATCATAC
Elongin B bait	N-38 cell cDNA	pCEL	BamHI- NotI	5'-CGCGGATCCGACGTGTTTCTCATGATCC -3' and 5'- CGCTGATCATCACTGCACAGCTTGTTC -3'
SOCS2 (LC-QQ) prey	pMG2-SOCS2		mutagenesis	5'GTATACATCAGCACCCACTCAGCAGCATTTCCAACGACTCGCC ATTAAC 5'GTTAATGGCGAGTCGTTGGAAATGCTGCTGAGTGGGTGCTGA TGTATAC
SOCS4 prey	mouse thymus cDNA	pMG2	EcoRI-NotI	5'GCGGAATTCGCTGAAAACAATAGT 5'CGCGCGGCCGCTCACTGCTGCTCTGGCA
SOCS5 prey	RZPD clone IRAV p968 D11111D6	pMG2	EcoRI-NotI	5'GCGGAATTCGATAAAGTGGGGAAAATGTG 5'CGCGCGGCCGCTTACTTTGCTTTGACTG
SOCS6 prey	RZPD clone IRAV p968 E0635 D6	pMG2	EcoRI-NotI	5'GCGGAATTCAAGAAAATCAGTCTG 5'CGCGCGGCCGCTCAGTAGTGCTTCTCC
SOCS7 prey	N-38 cell cDNA	pMG2	EcoRI-XbaI	5'GCGGAATTCGTGTTCCGCAACGTG 5'CGCTCTAGACTACGTGGAAGGCTCCA

Table 1: Overview of constructs used in this study
* Mutagenesis was used to eliminate an overlapping ORF.
** the BamHI site in the C-terminus of the leptin receptor (LR) was eliminated by mutagenesis.
*** SOCS7 was cloned in pZeroBlunt, cut with XbaI and blunted and subsequently partially digested with SacI. This allowed ligation in a pCEL vector which was digested with NotI, blunted and then partially digested with SacI.

Generation of the prey constructs pMG2-CIS and pMG2-SOCS2 both containing part of the gp130 chain (aa 905-aa 918) in duplicate was described earlier (37). The Epo receptor tyrosine 402 bait and pMET7- SV40 large T antigen (SVT) expression vectors were obtained as described previously (36).

Cell culture, transfection procedures and reagents

Hek293-T, 3T3-F442A and N-38 cells were cultured in 10% CO_2 humidified atmosphere at 37°C, and grown using DMEM (Gibco BRL) with 10% foetal calf serum (Cambrex). For transfection experiments, cells were freshly seeded in 6-wells plates. Hek293-T cells were 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. N-38 cells were transfected by using Lipofectamine 2000 (Invitrogen) following the manufacturer's guidelines. One day after transfection, cells were washed with PBS-A (PBS without calcium, magnesium and sodium bicarbonate), and cultured until further use. Recombinant mouse leptin and human erythropoietin (Epo) were purchased from R&D Systems. Human growth hormone was purchased from ImmunoTools and human IFNβ was generated in the laboratorium.

Luciferase and SEAP assays

For a typical luciferase experiment, Hek293-T or N-38 cells were transfected with the desired constructs together with a luciferase reporter gene. For STAT5 dependent luciferase assays, we used a β-casein–derived STAT5 luciferase reporter plasmid (38). For the STAT3 dependent luciferase experiments the pXP2d2-rPAPI-luciferase reporter, originating from the rPAPI (rat pancreatitis associated protein I) promoter, was used as previously described (36). 24 hours after transfection, cells were left untreated or were stimulated with ligand. After another 24 hours, luciferase activity from triplicate samples was measured by chemiluminescence in a Topcount luminometer (Canberra Packard). For the ISGF3 dependent SEAP assays we used the 6-16 SEAP reporter construct that was constructed as described previously (39). The amount of secreted alkaline phosphatase was determined with the phospha-light kit (Tropix, Bedford), using disodium 3(4-methoxyspiro(1,2-dioxetane-3,2'-

(5'chloro)tricycloneo[3.3.1.1]decan)-4-yl)phenyl phosphate (CSPD) as the luminogenic substrate. Assays were performed in a 96-well microtiterplate following the manufacturer's guidelines. Cells were lysed in a buffer (1% Triton X-100, 20 mM Tris pH 7,4) and alkaline phosphatase activity from triplicate samples was measured by chemiluminescence in a Topcount luminometer (Canberra Packard).

Western blot analysis and co-immunoprecipitation

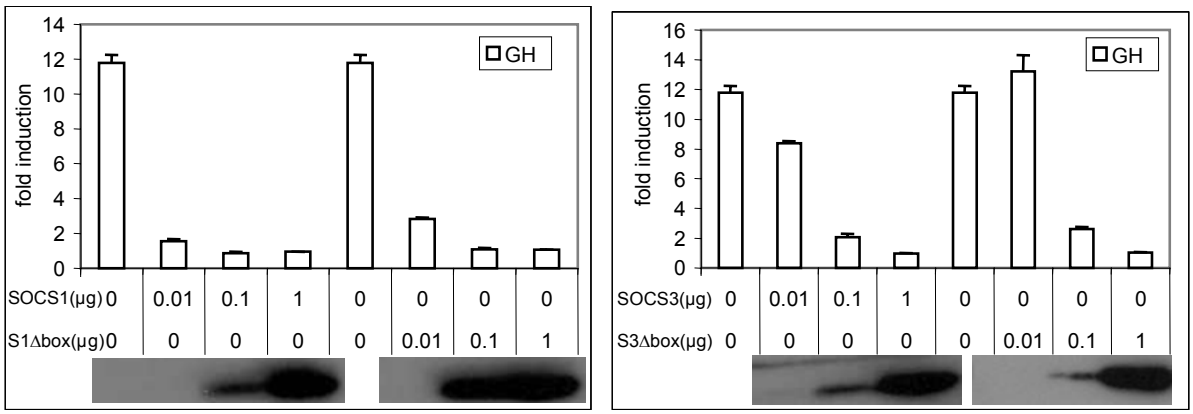
Transfected Hek293-T or N38 cells were lysed in modified RIPA buffer (200 mM NaCl, 50 mM Tris-HCl pH 8, 0,05% SDS, 2 mM EDTA, 1% NP40, 0,5% deoxycholic acid (DOC), 1 mM Na_3VO_4, 1 mM NaF, 20 mM β-glycerophosphate and Complete™ Protease Inhibitor Cocktail (Roche)). 5X loading buffer (156 mM Tris-HCl pH 6,8, 2% SDS, 25% glycerol, 0,01% Bromphenol blue sodium salt, 5% β-mercapto-ethanol) was added to the cell lysates which were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Blotting efficiency was checked using Ponceau S staining (Sigma). Blocking, washing and incubation with antibodies were carried out in TBS supplemented with: 5% dried skimmed milk and 0.1% Tween 20. 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 (Amersham Biosciences). Polyclonal rabbit anti-SOCS2 was a gift from Dr. Johnston and mouse β-actin antibody was supplied by Sigma. Immunoblots were then revealed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Amersham Biosciences) and SuperSignal West Pico Chemiluminescent Substrate (Pierce). For the co-immunoprecipitation experiments approximately 2 x 10^6 Hek293-T cells were transfected with Flag-tagged or E-tagged pMET7 SOCS expressionvectors. Cleared lysates (modified RIPA lysis buffer) were incubated with 4,0 µg/ml anti-FLAG mouse monoclonal antibody (Sigma) or anti E-tag mouse monoclonal antibody (Amersham Biosciences) and protein G-sepharose (Amersham Biosciences). After immunoprecipitation, SDS-PAGE and Western Blotting, interactions were detected using anti-Flag (Sigma) or anti-E-tag antibody (Amersham Biosciences) as described previously.

Results

An essential role for the SOCS2 SOCS-box to antagonise SOCS1 and SOCS3 inhibition of cytokine signalling.

SOCS2 exerts a dual action on growth hormone (GH) and prolactin (PRL) signalling 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 sub-domains. The effect of constitutive expression of SOCS proteins on GH signalling was investigated in Hek293-T cells using the STAT5-responsive β-casein luciferase reporter. Fig. 1A shows dose-response curves demonstrating complete inhibition of GH signalling by SOCS1 and SOCS3, respectively. GH-inducible activity is 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 with anti-Flag antibody. After removal of the SOCS-box of SOCS1 (SOCS1-Δbox) and SOCS3 (SOCS3-Δbox) inhibition was slightly reduced but not abolished in this assay system. Co-expression of SOCS2 could completely suppress the SOCS1- and SOCS3-dependent inhibition of GH signalling (Fig. 1B and 1C), whereby SOCS1 inhibition appeared to be more sensitive to the counteracting effect by SOCS2 than SOCS3. Of note, the amounts of SOCS1 used in this experiment could not be visualized by western blot analysis, indicating that working concentrations are approaching the physiological concentrations of this SOCS protein. The SOCS2 amounts also are not supra-physiological as the SOCS2 concentration at which a cross-regulatory effect is observed is comparable to the endogenous levels of SOCS2 in the growth hormone responsive 3T3-F442A mouse pre-adipocyte cell line (Fig. 1D). This suppressive effect on SOCS molecules is specific for SOCS2 as co-expression of CIS did not interfere with SOCS1 or SOCS3 mediated inhibition (data not shown). Strikingly, this SOCS2 effect strictly depended on the presence of its SOCS-box. It is of note that the deletion of the SOCS-box led to enhanced expression in case of SOCS2. This effect is also observed, albeit to a lesser extent with SOCS1, but is not observed for SOCS3.

Fig. 1A:



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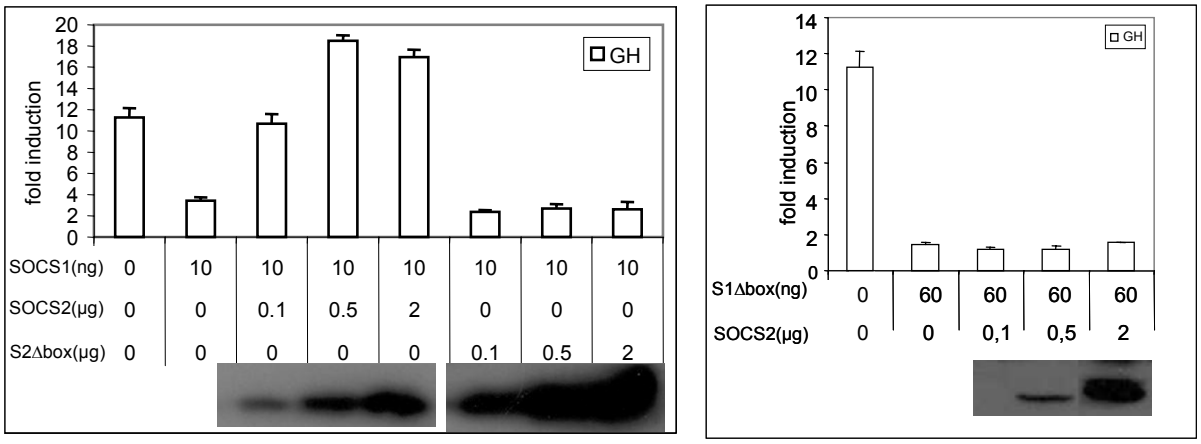

Fig. 1C:

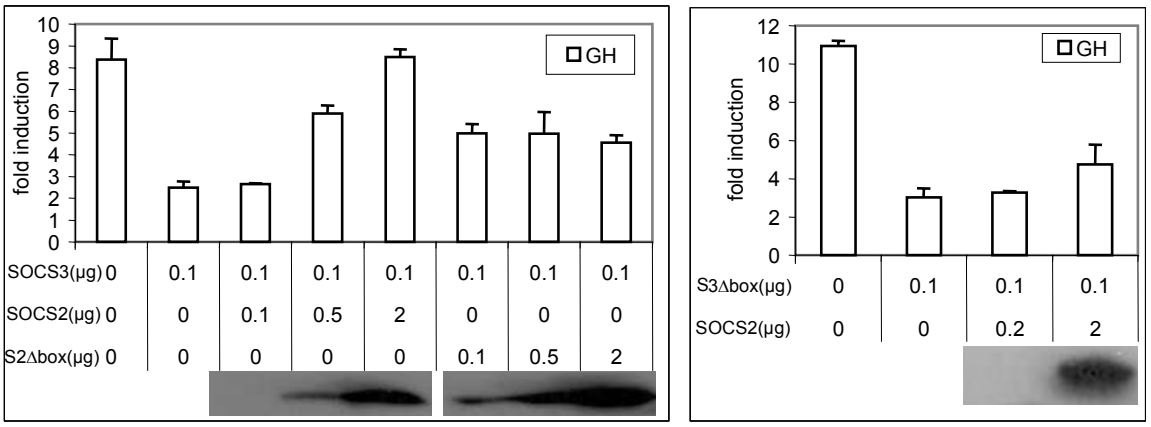

Fig. 1D:

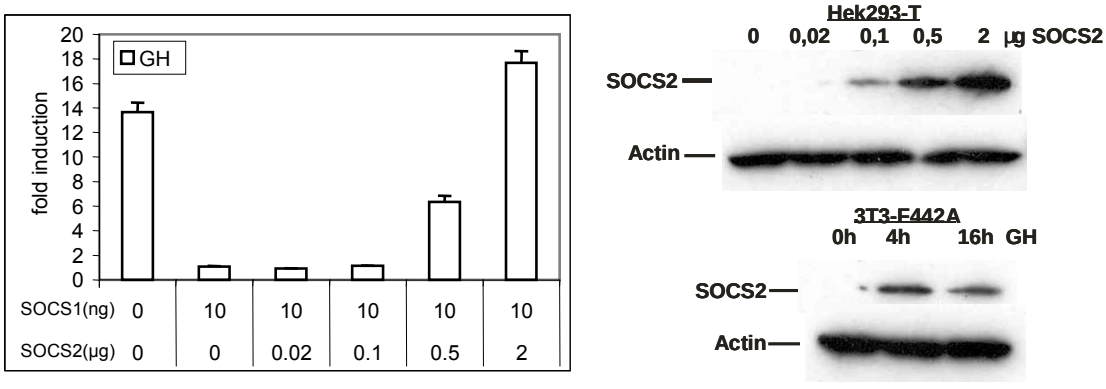

Figure 1: An essential role for the SOCS-box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of GH signalling.
Hek293-T cells were transfected with a rabbit GHR (40ng) expression vector and a β casein luciferase reporter gene (200ng). 24 hours after transfection, the cells were deprived of serum and then treated with human GH (200ng/ml) for 15 hours 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. A range of concentrations of SOCS1, SOCS3 or their SOCS-box deletion mutant plasmids (S1Δbox or S3 Δbox) were co-transfected to analyze inhibitory effect on GH signalling. A sample of lysate from each group was western blotted and probed with anti-Flag antibody.
B. A fixed amount of SOCS1 (10ng) or SOCS1 Δbox (60ng) was co-transfected with increasing concentrations of SOCS2 or SOCS2 Δbox (S2Δbox). Expression of the E-tagged SOCS2 and SOCS2 Δbox proteins in the same transfected cells was verified on lysates using anti-E antibody.
C. A fixed amount of SOCS3 (100ng) or SOCS3 Δbox (100ng) was co-transfected with increasing concentrations of SOCS2 or SOCS2 Δbox. Expression of the E-tagged SOCS2 and SOCS2 Δbox proteins in the same transfected cells was verified on lysates using anti-E antibody.
D. Comparison of ectopic and endogenous expression levels of mouse SOCS2 in respectively Hek293-T and 3T3-F442A. A fixed amount of SOCS1 (10ng) was co-transfected with increasing concentrations of SOCS2 in Hek293-T. Expression of the E-tagged SOCS2 proteins in the same transfected cells and of the endogeneous SOCS2 in the 3T3-F442A cells was verified on lysates using anti-SOCS2 antibody. 3T3-F442A were incubated in serum-free medium prior to stimulation with GH (200ng/ml) for the indicated times. Levels of loaded protein were normalised by determining the protein concentrations with the Bradford method and this was verified by Ponceau S staining. As additional control, the blots were stripped and probed with β-actin antibody to check for equal loading of cell lysates of the same cell type.

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 (IFNaR) signalling (40,41), however, the role of SOCS2 is less well elucidated. We monitored interferon-β (IFN-β) signalling in Hek293-T cells using the type I interferon-sensitive 6-16 SEAP 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 signalling (Fig. 2A): at low concentrations, SOCS2 suppressed IFN signalling, but higher concentrations of SOCS2 lead 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 or SOCS3 or of their mutants lacking the SOCS-box can inhibit IFN-β signalling (Fig. 2B). Again analogous to the observations made for GH, SOCS1 and SOCS3-mediated inhibition of IFN-β signalling could be completely neutralized by co-expression of SOCS2, and the SOCS-box of SOCS1 or -3 and of SOCS2 is strictly required for the full effect (Fig. 2C and 2D).

Fig. 2A :

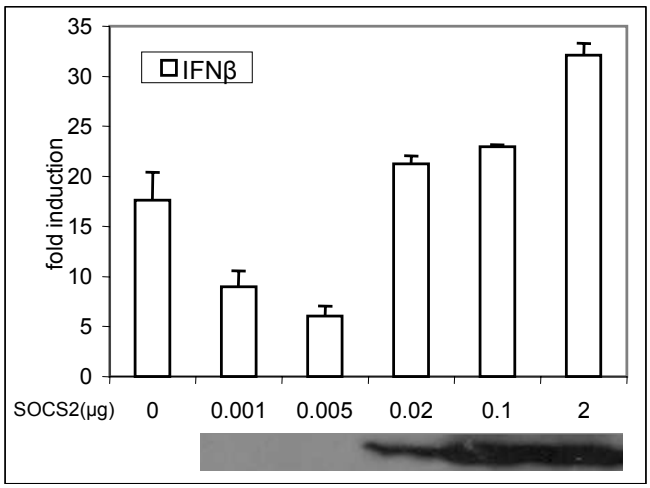

Fig. 2B :

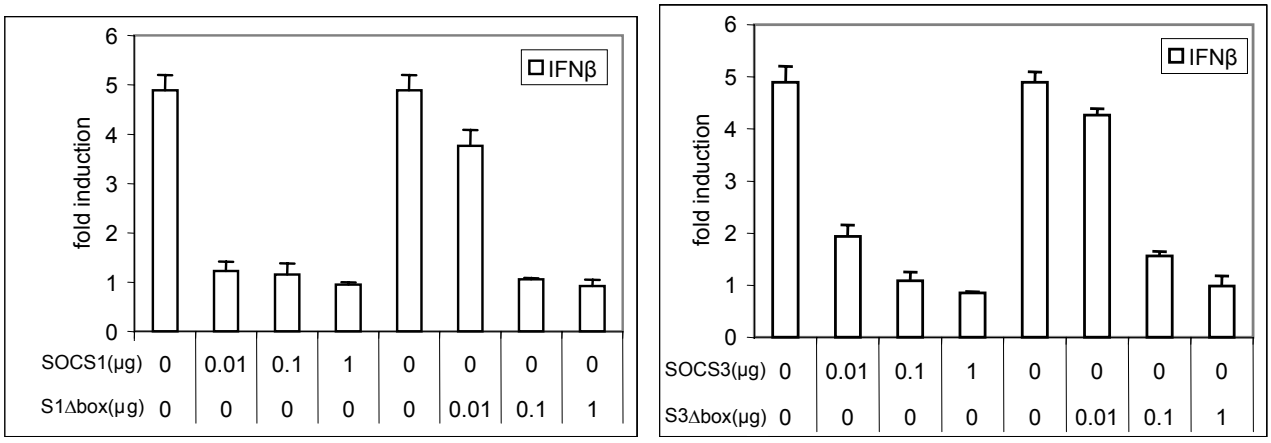

Fig. 2C :

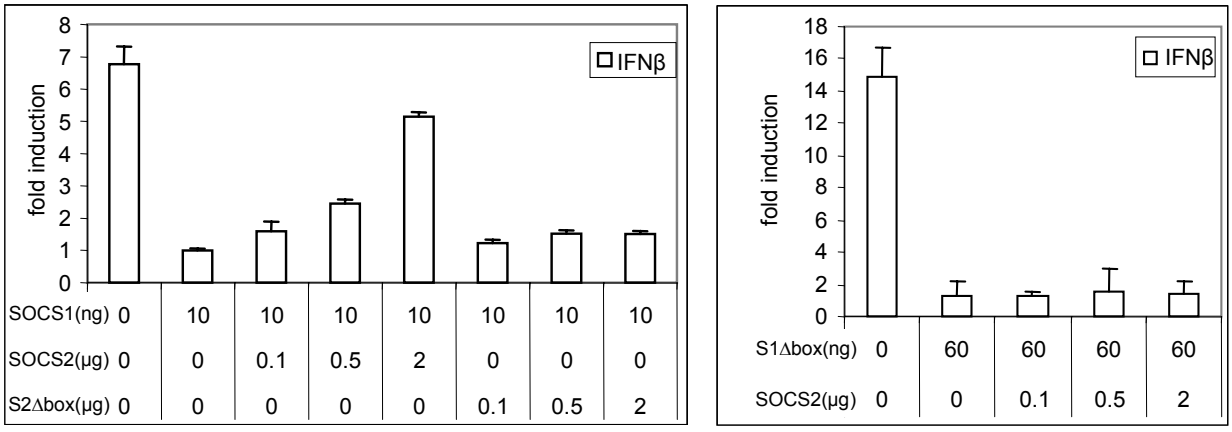

Fig. 2D :

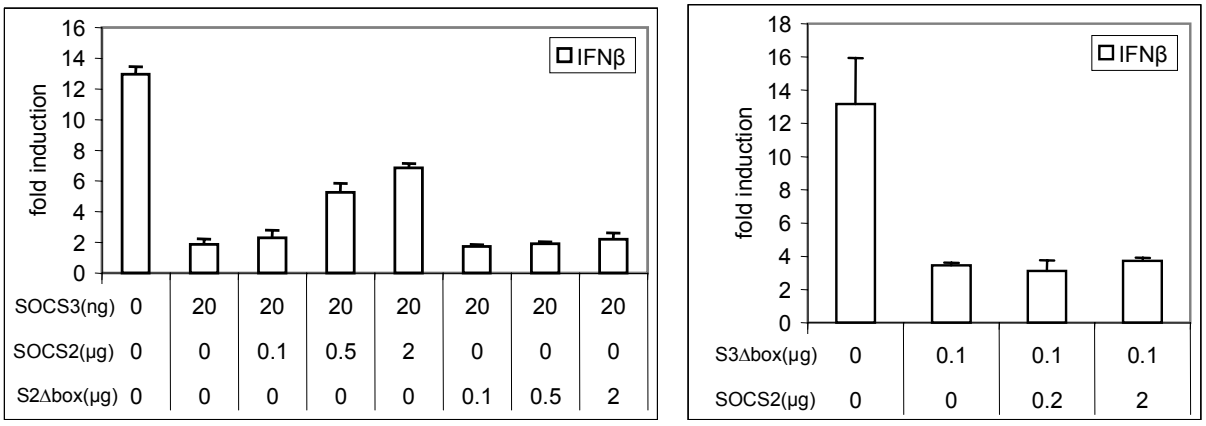

Figure 2: An essential role for the SOCS-box in interference of SOCS2 with SOCS1 and SOCS3 inhibition of interferon signalling.
Secreted alkaline phosphatase (SEAP) activity was assayed in Hek293-T cells transfected without SOCS or with several ratios of SOCS1, -2 or -3 expression vectors and the IFN-responsive 6-16 SEAP reporter (200 ng). After 24 hours, transfected cells were stimulated with human IFN β (100pM) and the SEAP activity from the 6-16 reporter gene was measured after 48 hours. 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 on interferon type I signalling as assessed by transfecting a range of concentrations of SOCS2 gene or the appropriate amount of empty vector in Hek293-T cells. Expression of the E-tagged SOCS2 protein in the same transfected cells was verified on lysates using anti-Etag antibody.
B. A range of concentrations of SOCS1, SOCS3 or their SOCS-box deletion mutants plasmids were transfected to analyze inhibitory effect on interferon type I signalling.
C. A fixed amount of SOCS1 (10ng) or SOCS1 Δbox (60ng) was co-transfected with increasing concentrations of SOCS2 or SOCS2 Δbox.
D. A fixed amount of SOCS3 (20ng) or SOCS3 Δbox (100ng) was co-transfected with increasing concentrations of SOCS2 or SOCS2 Δbox.

We finally extended these analyses of SOCS modulation also to leptin receptor (LR) signalling. Again quite similar to the previous observations, expression of SOCS1, SOCS3 or their SOCS-box deletion mutants blocked induction of leptin-mediated activation of a STAT3-responsive rat Pancreatitis Associated Protein I (rPAPI) promoter-luciferase reporter in Hek293-T cells (data not shown). Co-expression of SOCS2 could restore the SOCS-dependent signalling blockade. Those effects were dependent on either SOCS-box and were less pronounced for SOCS3 mediated LR inhibition than for SOCS1 (data not shown). We further verified the cross-modulatory effects of SOCS2 in mouse hypothalamic N38 cells which represent a physiological context for leptin receptor signalling. 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 or SOCS3 or of their mutants lacking the SOCS-box can inhibit leptin signalling (Fig. 3A). Co-expression of SOCS2 with SOCS1 (Fig. 3B) and SOCS3 (data not shown) in the N-38 cells led to recovery of the leptine-induced signalling in a SOCS-box dependent manner. Moreover, expression of SOCS2 alone clearly stimulated 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.

Fig. 3A:

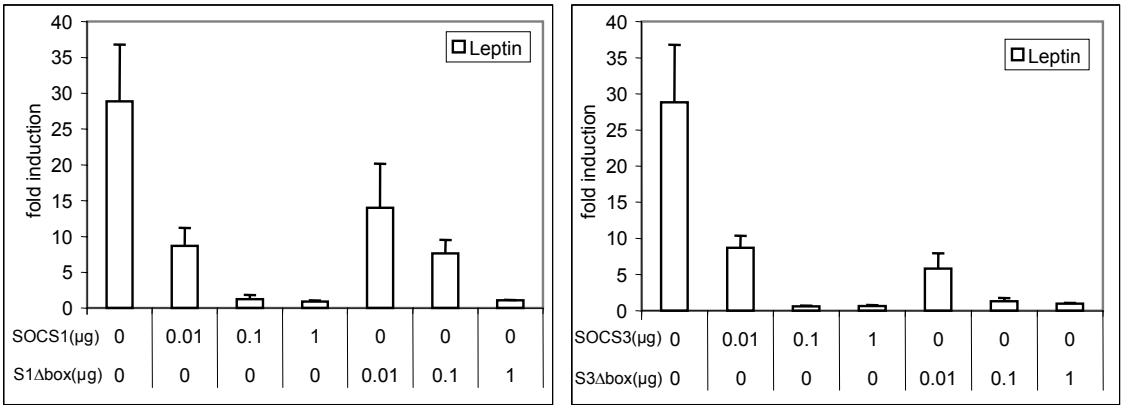

Fig. 3B:

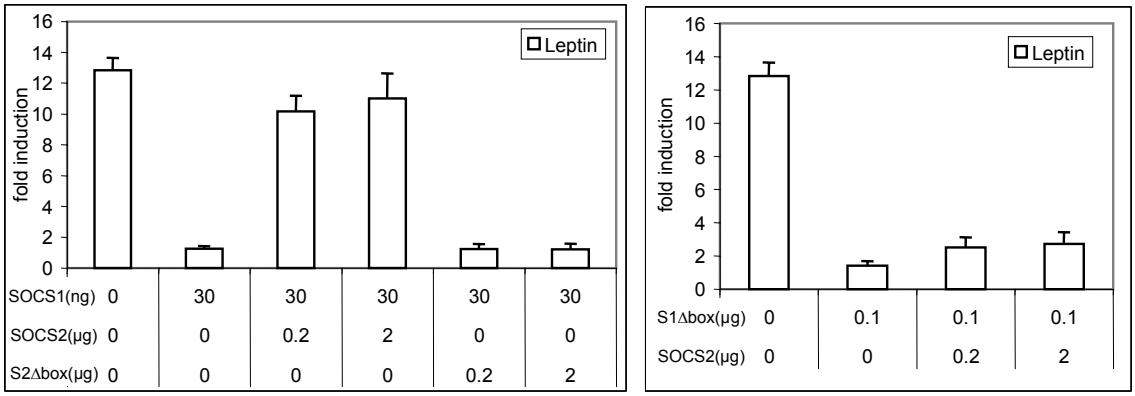

Fig. 3C:

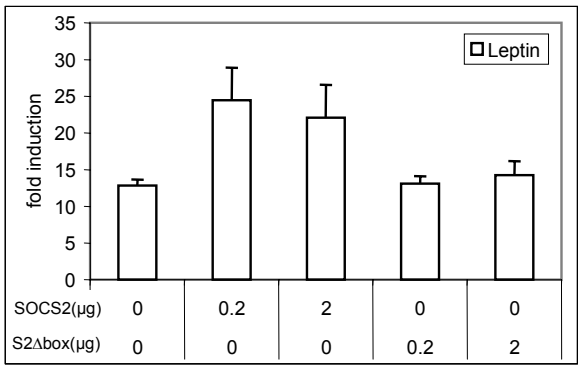

Figure 3: SOCS2 displays a SOCS-box dependent stimulatory effect on leptin signalling in N38 cells
Mouse hypothalamic N38 cells were transiently co-transfected with a mouse LR (250ng) expression vector and the pXP2d2-rPAP1-luci reporter (1µg). The transfected cells were either stimulated for 24 h with leptin (100ng/ml) or were left untreated. Luciferase measurements were performed in triplicate. Data are expressed as ratio leptin stimulated/non stimulated.
A. A range of concentrations of SOCS1, SOCS3 or their SOCS-box deletion mutant plasmids were co-transfected to analyze inhibitory effect on leptin signalling.
B. A fixed amount of SOCS1 (30 ng) or SOCS1 Δbox (100 ng) was co-transfected with increasing concentrations of SOCS2 or SOCS2 Δbox.
C. A range of concentrations of SOCS2 or the SOCS2 Δbox mutant plasmids were transfected to analyze stimulatory effect on leptin signalling.

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/SL/MxxxC/SxxxV/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 which abrogate elongin BC recruitment (9). In another SOCS2 derivative, SOCS2 (LC-QQ), both residues were mutated to glutamines 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 signalling in Hek293-T cells and with leptin signalling 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 on other SOCS proteins.

SOCS2 interacts with other members of the SOCS family.

We next used MAPPIT (Mammalian Protein-Protein Interaction Trap), 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 erythropoietin receptor (EpoR) linked to the transmembrane and the intracellular part of a signalling deficient LR. The use of a triple Y to F mutant leptin receptor (further referred to as LR-F3) knocks out STAT3 activation, and offers the added advantage that negative feedback mechanisms are inoperative, implying enhanced signalling.

MAPPIT prey constructs are composed of a prey protein 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 and induction of the STAT3-responsive rPAPI 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 manuscript is shown in Fig 5A.

Fig. 4A :

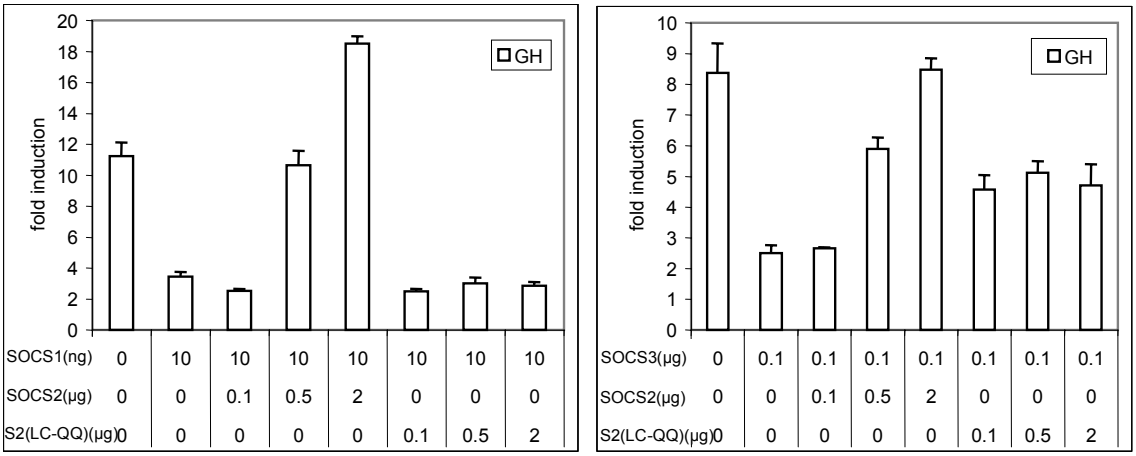

Fig. 4B :

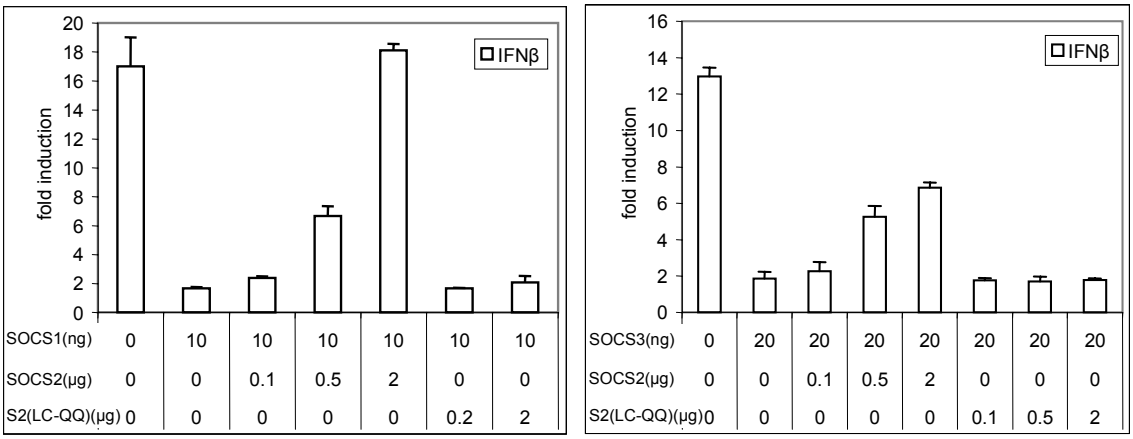

Fig. 4C :

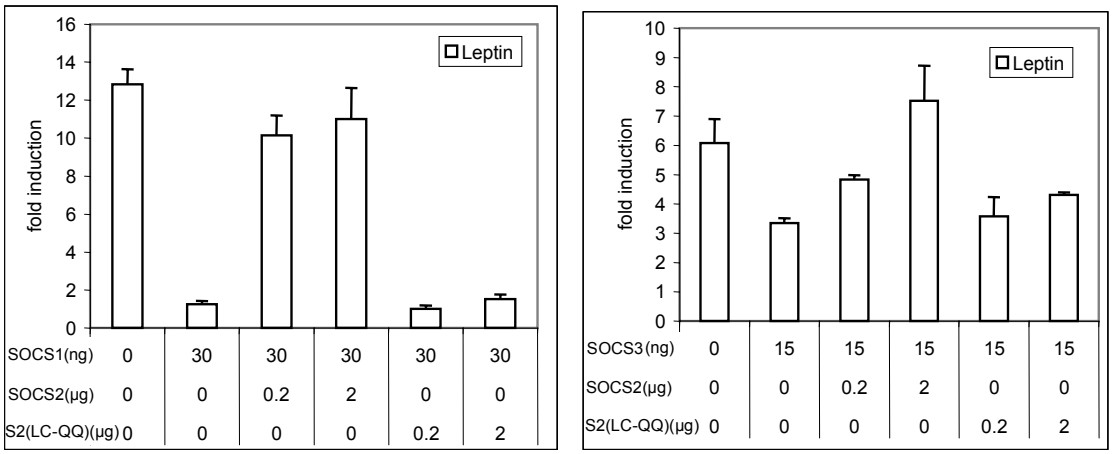

Figure 4: The negative effect of SOCS2 on other SOCS proteins is dependent on recruitment of the elongin BC complex

Hek293-T or N38 cells were transiently transfected with fixed amounts of plasmids encoding SOCS1 or SOCS3 and increasing concentrations of SOCS2 *wild type* or SOCS2 ΔElongin BC (LC-QQ). The transfected cells were either stimulated with hGH (200ng/ml), IFN β (100pM) or leptin (100ng/ml) or were left untreated. Luciferase and SEAP measurements were performed in triplicate. Data are expressed as fold induction (stimulated/non stimulated).

A. Hek293-T cells were transfected with 10ng SOCS1, 100ng SOCS3 and increasing concentrations of SOCS2 derivatives. GH signalling was assayed as described in Fig.1

B. Hek293-T cells were transfected with 10ng SOCS1, 20ng SOCS3 and increasing concentrations of SOCS2 derivatives. IFN signalling was assayed as described in Fig.2

C. N38 cells were transfected with 30ng SOCS1, 15ng SOCS3 and increasing concentrations of SOCS2 derivatives. Leptin signalling was assayed as described in Fig.3

Fig. 5A :

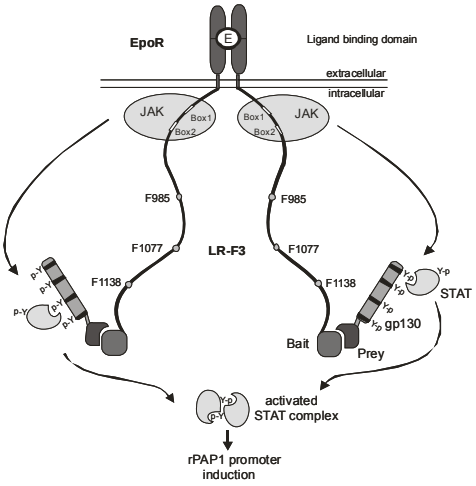

Fig. 5B :

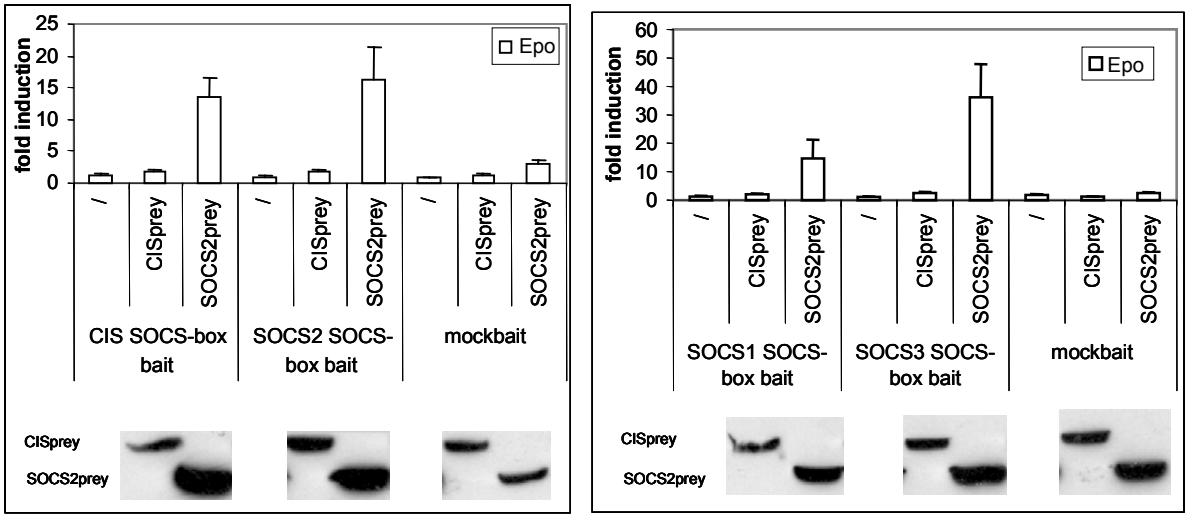

Fig. 5C :

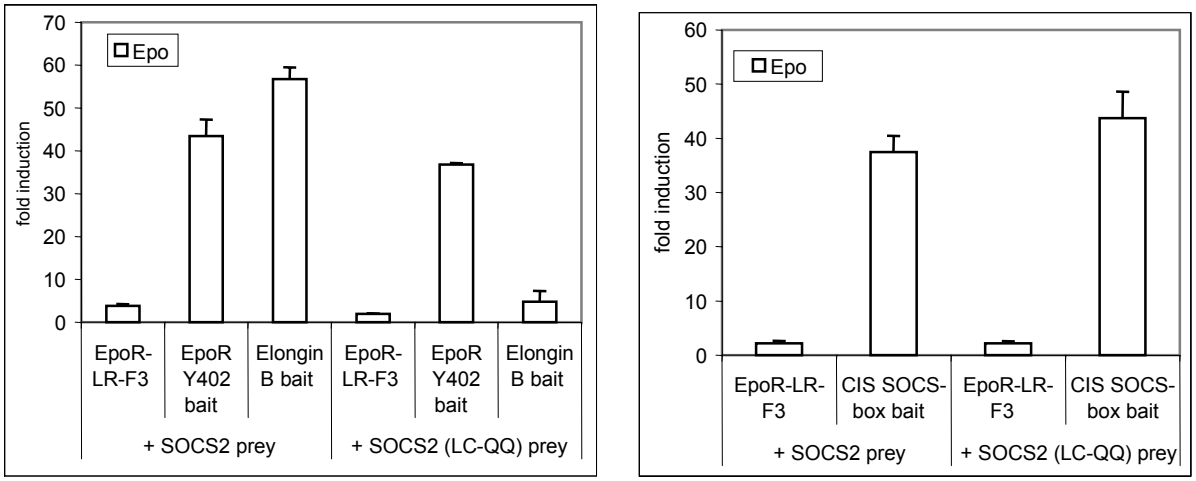

Figure 5: MAPPIT analysis of SOCS interactions
A. Principle of MAPPIT
See results section for details.
B. SOCS2 interacts with the SOCS-box of SOCS1, -2, -3 and CIS
Hek293-T cells were transiently co-transfected with plasmids encoding bait variants with the SOCS-box of several SOCS proteins or with a mock bait lacking the SOCS motif, the pMG2-SOCS2 and pMG2-CIS prey-construct and with the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. 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 while the interaction with CIS is preserved
Hek293-T cells were transiently co-transfected with plasmids encoding the chimeric EpoR-LR-F3 construct as a negative control, the EpoR Y402 bait as positive control for SOCS2, the elongin B bait or CIS SOCS-box bait, and the pMG2-SOCS2 or pMG2-SOCS2 (LC-QQ) prey constructs, combined with the pXP2d2-rPAP1-luciferase. The transfected cells were either stimulated for 24 hours with Epo or were left untreated. Luciferase measurements were performed in triplicate. Data are expressed as fold induction (stimulated/not stimulated).

We have previously shown that SOCS2 directly interacts with CIS (34). This observation and the abovementioned findings on cross-regulation between SOCS2 and SOCS1 and -3 prompted us to investigate whether SOCS2 could bind to those SOCS family members. Hek293-T cells were co-transfected with a bait plasmid encoding the SOCS-box of either SOCS1, -2, -3 or CIS combined with a plasmid encoding the SOCS2 prey or CIS prey and the STAT3-responsive luciferase reporter construct. We always used isolated SOCS-boxes as bait proteins since in case of SOCS1 and SOCS3 the full-length baits did interfere with the MAPPIT read-out and therefore could not be investigated. Epo stimulation revealed clear interaction of SOCS2 with all baits examined. In contrast, the CIS prey failed to induce any reporter activity (Fig. 5B). Expression levels of the Flag-tagged prey proteins were confirmed by immunoblotting using an 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 Y402 motifof the EpoR was used as a positive control as this receptor motif directly interacts with SOCS2 (36). This association of SOCS2 with SOCS1, SOCS2, SOCS3 and CIS was confirmed by co-immunoprecipitation. We transiently co-transfected Hek293-T cells with a plasmid encoding E-tagged SOCS2, together with Flag-tagged plasmids encoding respectively SOCS1, SOCS2 and SOCS3 or we co-expressed Flag-tagged SOCS2 with E-tagged SOCS1, SOCS2 and CIS. SOCS2 could co-immunoprecipitate SOCS1 to -3 (Fig. 6A) and in case of CIS, both the 37- and 32-kDa forms that correspond to mono- and non-ubiquitinated forms respectively (44). Observed interactions of SOCS2 with SOCS proteins in Hek293-T

cells depended on proteasomal inhibition with the proteasomal inhibitor MG132 (20µM) for 6 hours and stimulation with IFNβ (100pM) for 30 min. SOCS1 could still be co-immunoprecipitated with the SOCS2 Δbox and SOCS2 (LC-QQ) mutant, indicating that the 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 weakens the interaction with SOCS1, suggesting a role for this domain in SOCS-SOCS interactions.

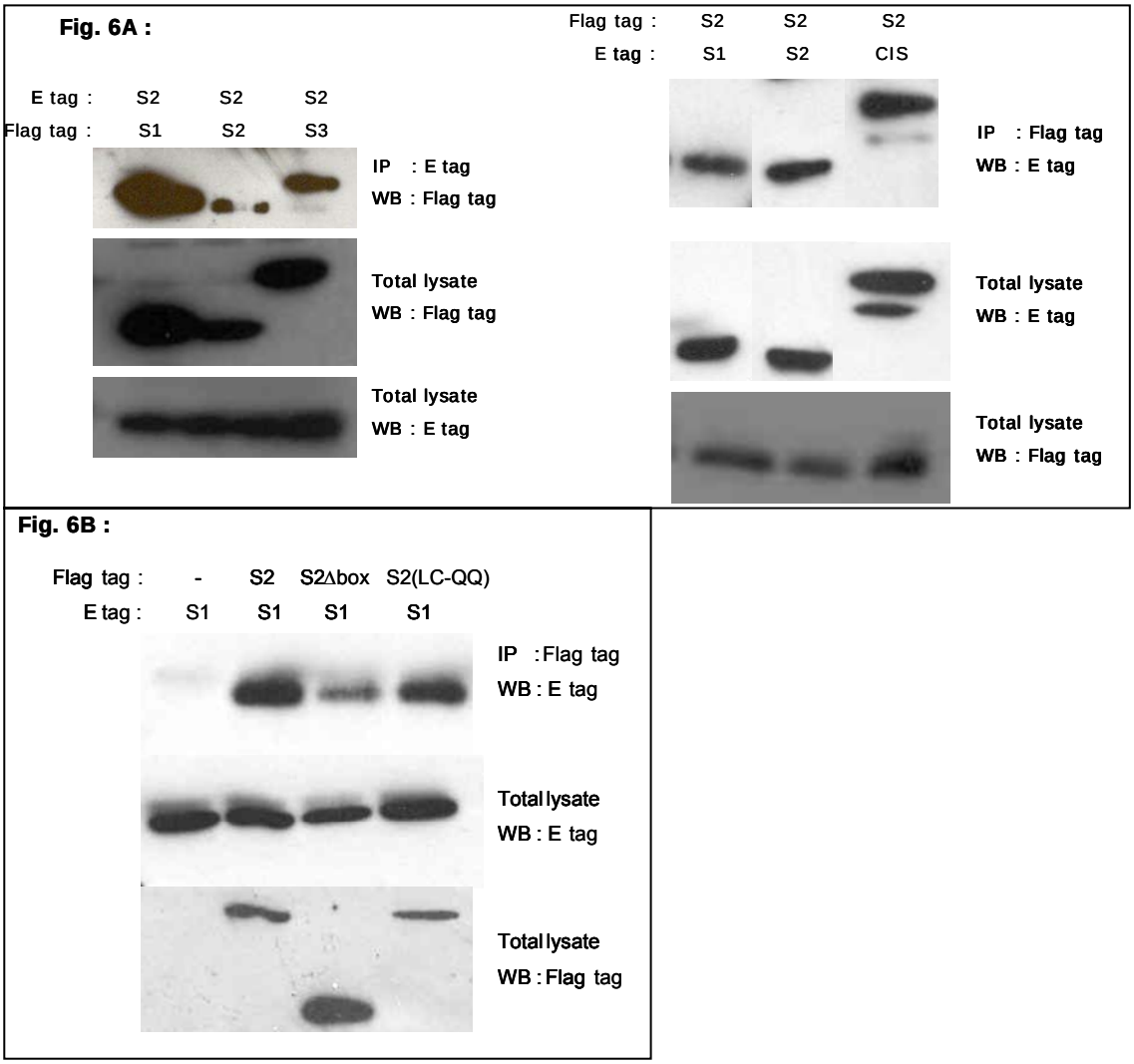

Figure 6:
A. Interactions of SOCS2 demonstrated by co-immunoprecipitation experiments
Lysates from Hek293-T cells co-transfected with Flag-tagged or E-tagged SOCS1 (S1), SOCS2 (S2), SOCS3 (S3) and CIS were immunoprecipitated with anti-E or anti-Flag antibodies and western blotted with anti-Flag or anti-E antibodies (Top). Whole cell lysate was western blotted with anti-Flag or anti-E antibodies as loading controls (middle and bottom).
B. Interaction analysis of SOCS1 and SOCS2 mutants
Hek293-T cells were transiently co-transfected with Flag-tagged SOCS2, SOCS2 Δbox (S2Δbox), SOCS2 ΔElongin BC (LC-QQ) (S2(LC-QQ)) or the appropriate amount of empty vector and E-tagged SOCS1. Cell lysates were immunoprecipitated (IP) with Flag antibody and were subsequently immunoblotted (IB) with anti E tag or anti Flag.

SOCS2 promotes degradation of SOCS1.

The dependency 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 were treated with the protein synthesis inhibitor cycloheximide (20µM) for 6 hours. Degradation of SOCS1 was observed when increasing concentrations of SOCS2 were co-expressed whereas SOCS2 (LC-QQ) had no effect (Fig. 7). This suggests a mechanism in which SOCS2 acts as an adapter molecule between an E3 ubiquitin ligase complex and SOCS proteins targeting them for proteasomal turn-over.

Fig. 7 :

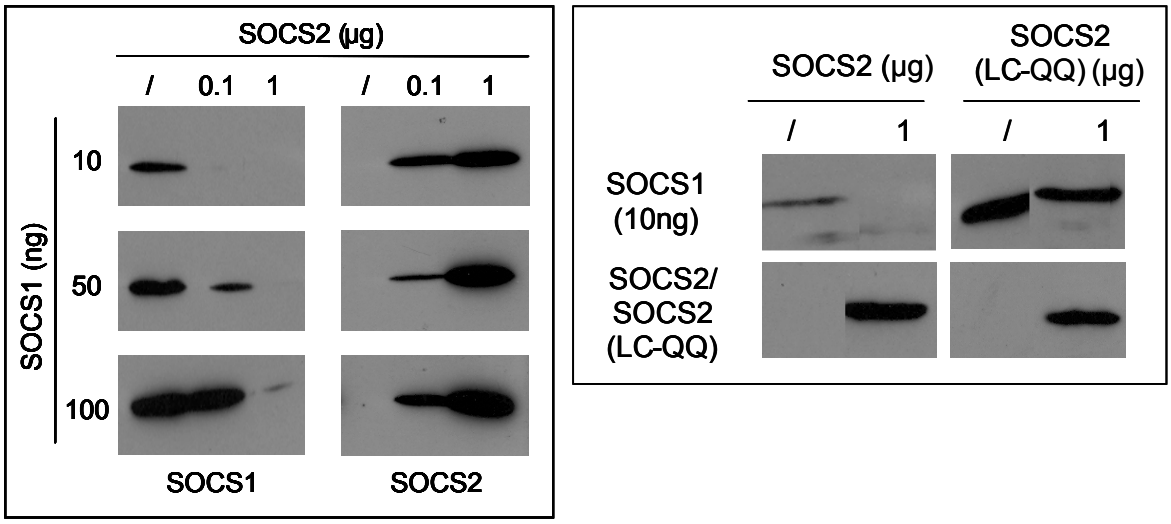

Figure 7: Co-expression of SOCS2 accelerates the SOCS1 degradation
Increasing concentrations of E-tagged SOCS2 or SOCS2 (LC-QQ) were transiently co-expressed in Hek293-T cells with Flag-tagged SOCS1. Cells were treated with cycloheximide (20µM) for up to 8 h. The lysates were blotted for SOCS1 with an anti-Flag antibody and for SOCS2 with an anti-E antibody.

SOCS2 but also SOCS6 and SOCS7 interact with all members of the SOCS family.

Interaction studies performed with the other SOCS proteins used as bait revealed that the SOCS2 prey can also interact with the SOCS-box of SOCS4, -5, -6 and -7 (Fig. 8A). Using the same approach we performed a matrix-type interaction analysis between SOCS proteins and we found that SOCS6 and SOCS7 preys also interact with the SOCS-box baits of all members of the SOCS family (Fig. 8B and 8C). Of note, SOCS2, -6 and -7 also display binding on themselves. The MAPPIT data with the CIS prey are included as negative control and functionality of this CIS prey was

Fig. 8A :

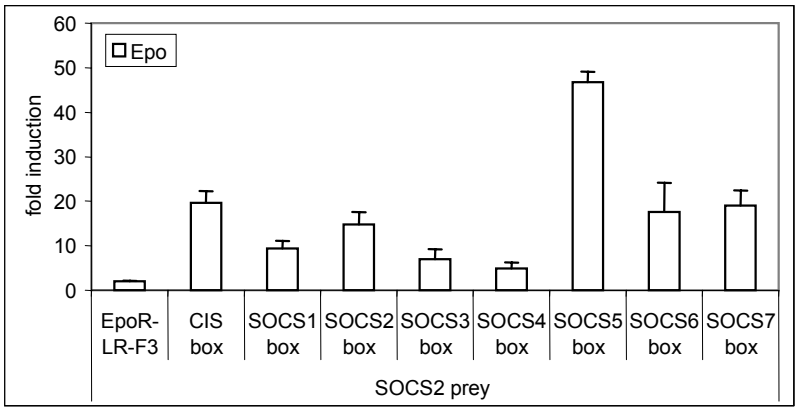

Fig. 8B :

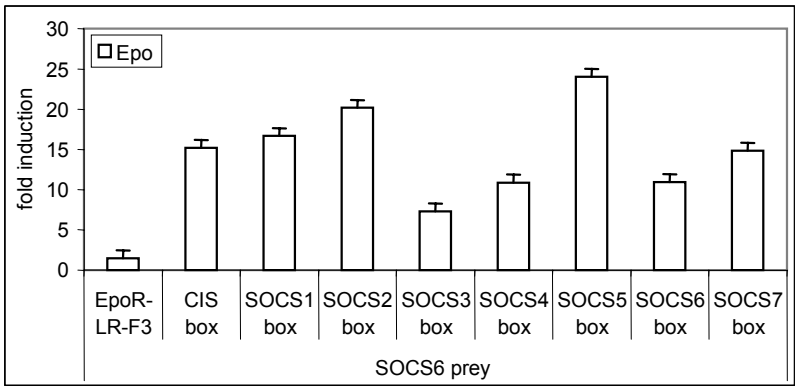

Fig. 8C :

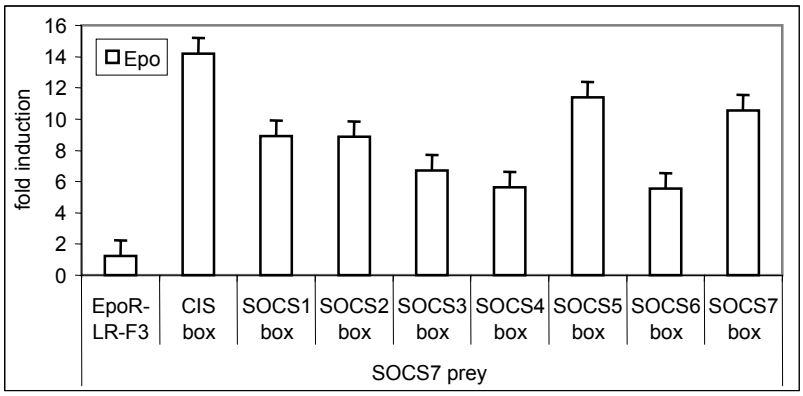

Fig. 8D :

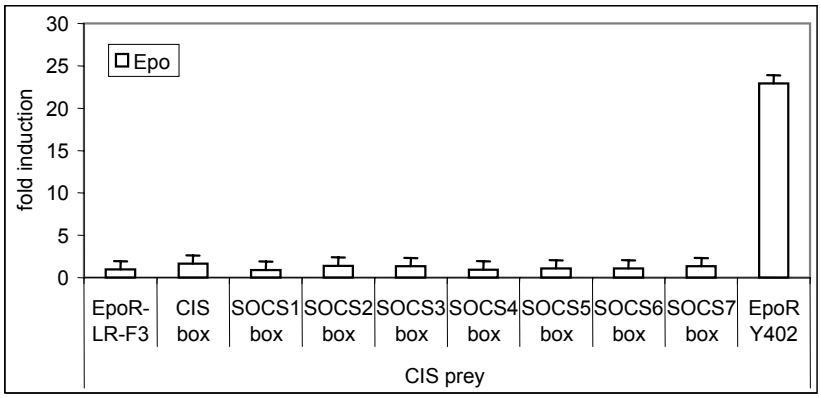

Figure 8:
A.-D. SOCS2, -6 and -7 interact with the SOCS-box of all SOCS members
Hek293-T cells were transiently co-transfected with plasmids encoding bait variants of the SOCS-box of all SOCS proteins or the chimeric EpoR-LR-F3 construct as a negative control, the pMG2-SOCS2, -6, -7 and CIS preys and the pXP2d2-rPAP1-luciferase reporter. After transfection, cells were either left untreated or were stimulated with Epo for 24 hours. Luciferase activities were measured in triplicates. Data are expressed as fold induction (stimulated/not stimulated).

Fig. 9A :

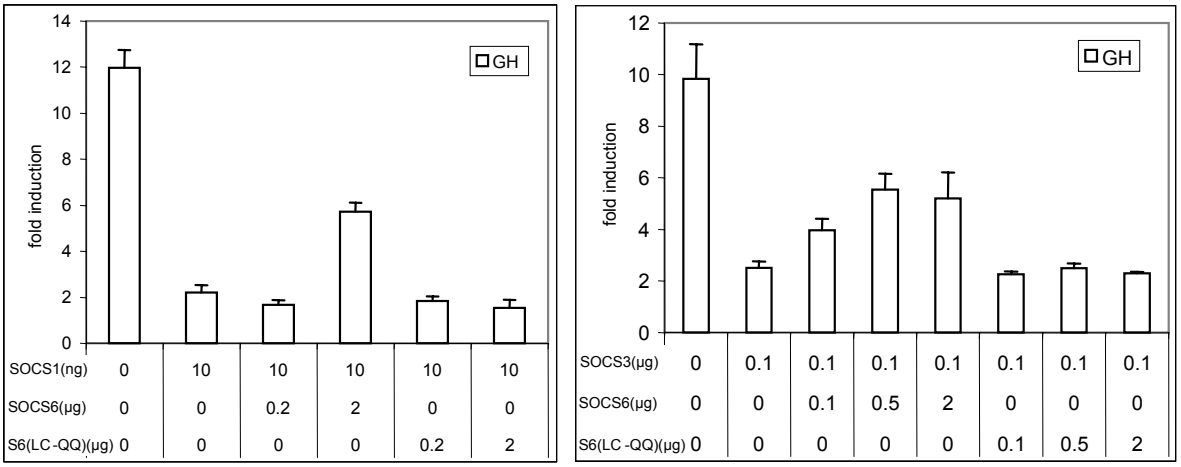

Fig. 9B :

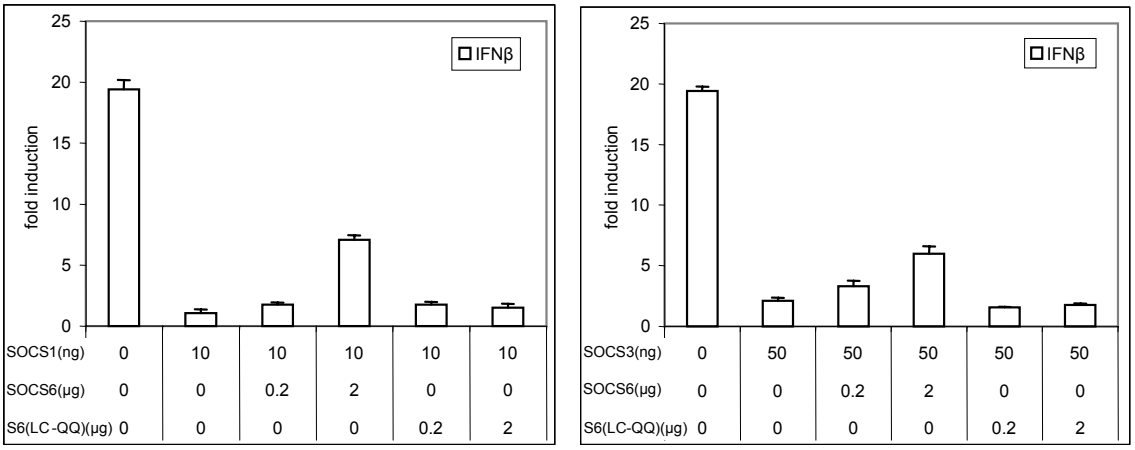

Fig. 9C :

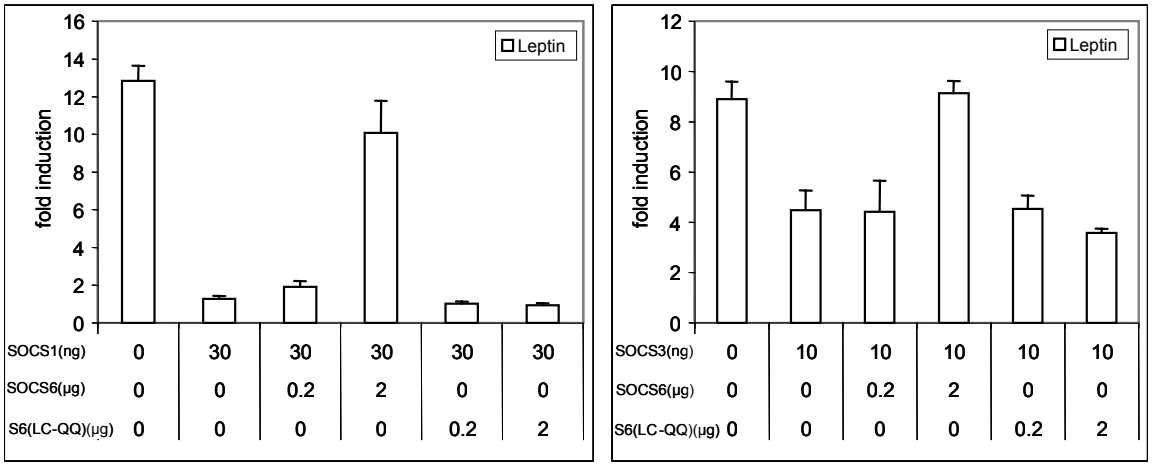

Figure 9: The negative effects of SOCS6 on other SOCS proteins depends on recruitment of the elongin BC complex

Hek293-T or N38 cells were transiently transfected with fixed amounts of plasmids encoding SOCS1 or SOCS3 and increasing concentrations of SOCS6 *wild type* or SOCS6 ΔElongin BC (LC-QQ). The transfected cells were either stimulated with GH (200ng/ml), IFN β (100pM) or leptin (100ng/ml) or were left untreated. Data are expressed as fold induction (stimulated/non stimulated). Luciferase and SEAP measurements were performed in triplicate.

A. Hek293-T cells were transfected with 10ng SOCS1, 100ng SOCS3 and increasing concentrations of SOCS6 derivatives. GH signalling was assayed as described in Fig.1

B. Hek293-T cells were transfected with 10ng SOCS1, 50ng SOCS3 and increasing concentrations of SOCS6 derivatives. IFN signalling was assayed as described in Fig.2

C. N38 cells were transfected with 30ng SOCS1, 10ng SOCS3 and increasing concentrations of SOCS6 derivatives. Leptin signalling was assayed as described in Fig.3

demonstrated using the interaction with the EpoR Y402 motif as a control (Fig. 8D). The EpoR-LR-F3 bait provided a control for aspecific binding on the intracellular part of the leptin receptor and JAK2. The expression of the different bait constructs was verified by checking the interaction with the SH2-β prey which binds the associated JAK of the 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 co-expression with an established MAPPIT interaction had no deleterious effect (data not shown). From these experiments we conclude that SOCS2, SOCS6 and SOCS7 can interact with the SOCS-box of all SOCS members.

Fig. 10 :

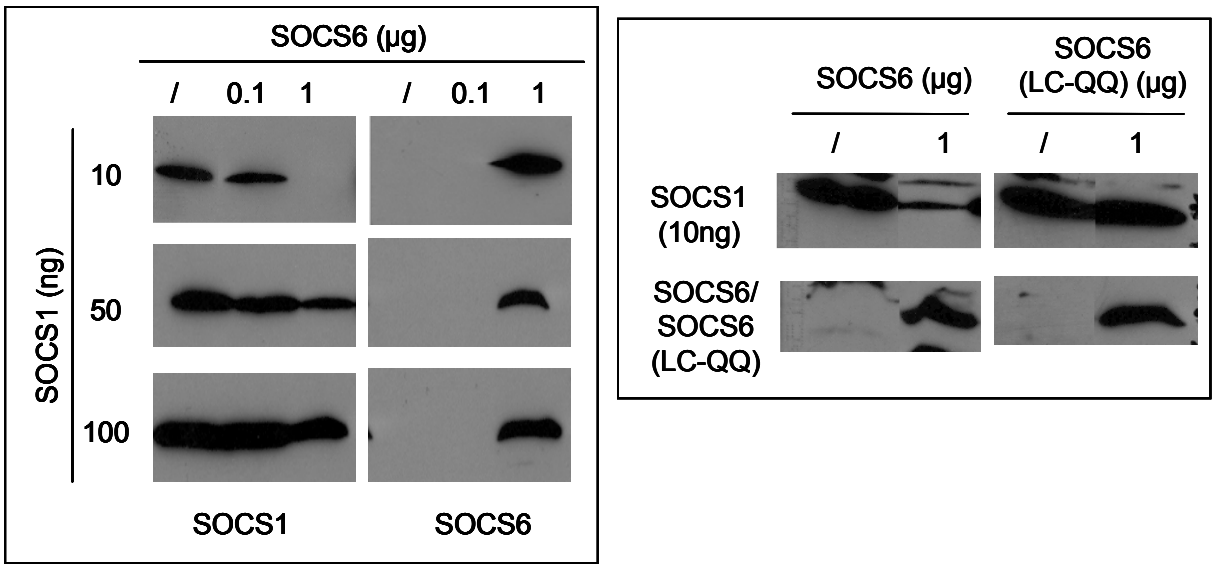

Figure 10: SOCS6 but not SOCS6 (LC-QQ) promotes degradation of SOCS1
Increasing concentrations of Flag-tagged SOCS6 or SOCS6 (LC-QQ) were co-expressed transiently in Hek293-T cells with Flag-tagged SOCS1. Cells were treated with cycloheximide (20µM) for up to 8 h. The lysates were blotted for SOCS1 and SOCS6 expression with an anti-Flag antibody.

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, IFN (Fig. 9A and 9B) and leptin (not shown) signalling in Hek293-T cells. In N38 cells, we could demonstrate that SOCS6 interfered with SOCS1 and SOCS3 inhibition of leptin signalling (Fig. 9C).

Like other SOCS molecules, SOCS6 was shown to bind to elongin 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, SOCS6 wild type, but not the ΔBC box mutant, reduced SOCS1 expression in a dose dependent manner (Fig. 10), indicating that SOCS6 mediates the observed inhibition by accelerating turn-over of other SOCS proteins. 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: E1 ubiquitin activating enzymes, E2 carrier enzymes and a large set of E3 ubiquitin ligases, whereby the latter define substrate specificity. The SCF (Skp1-Cullin-F-box) E3 ubiquitin ligase complex is composed of the Cul1 scaffold protein that binds the Roc1/Rbx1 RING domain protein and the E2 carrier enzyme, and that recruits, via the Skp1 linker protein, F-box proteins that 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 (VHL) and Cul5-SOCS-box adaptor proteins, whereby elongins BC and SOCS or VHL proteins fulfil the role of the Skp1 and F-box protein moieties, respectively (1). Evidence that SOCS proteins can mediate proteasomal turn-over of target molecules is accumulating. Examples include the GHR and EpoR (44,46), JAK2 (13), the Rac guanine nucleotide exchange factor Vav (47), Ras GAP (17) and insulin receptor substrate (IRS)1 and IRS2 (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 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 negative regulator of GH signalling in vivo and in vitro (43), but can also enhance GH signalling 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) or 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 model, and recently, Greenhalgh et al., showed that SOCS2 binds at Y487 and Y595 of the GH receptor, which are not usual immunoreceptor tyrosine-based inhibitory motifs, suggesting that competition of SOCS3 at these sites is not involved (43).

The key finding in this report 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 signalling by antagonizing SOCS1 and SOCS3 in a SOCS-box dependent manner. This effect is not limited to the GH system, since we found similar effects on signalling via the endogenous IFN type I receptor and leptin receptor, 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, co-expression of SOCS2 leads to lowered expression levels of this target SOCS protein. The critical elongin BC dependency of the inhibitory effect by SOCS2 strongly argues that SOCS2 functions as part of an E3 ligase complex. Alternatively, higher expression levels of SOCS2 may compete for recruitment of the elongin BC complex, indirectly leading to destabilisation of other SOCS proteins lacking this complex (vide supra). However, SOCS1 and -3 proteins lacking their entire SOCS-box were still able, although to a lesser extent, to inhibit cytokine signalling, but were completely refractory to the SOCS2 effect, implying that SOCS2 binding is critical. Moreover, overexpression of CIS that is equally well capable of sequestering elongin BC complexes does not lead to any effect on other SOCS proteins. Where examined, the SOCS-box of the target SOCS protein appeared to be involved in SOCS2 binding. Although our data support an involvement of the SOCS-box in the interaction between the inhibitory SOCS and the targetted SOCS, 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 Tannahill et al. by demonstrating SOCS2 regulation of the SOCS3-dependent inhibition of IL-2 and -3 signalling (29) and by Lavens et al. (34) showing elongin BC-dependent interference of SOCS2 with binding of CIS at position Y985 of the leptin receptor. In line with such regulatory role of SOCS proteins is the sequential induction pattern of different SOCS molecules. Unlike SOCS1 and SOCS3 that are typically induced in a rapid and transient manner upon cytokine stimulation, SOCS2 expression usually occurs late after cytokine 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,-6 and -7 can also bind to them selves, suggesting the possibility of self-elimination. A full and global insight in the precise inhibitory effects will thus require careful analyses 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 through 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. This interaction map shows 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 could show that its expression potentiates signalling via the GHR, IFNaR and LR, in a way quite similar to what we observed for SOCS2. Similar datasets were also obtained for leptin signalling in the physiologic relevant hypothalamic N38 cell line. These novel findings on SOCS6 provide an explanation for the significant enhancement in glucose metabolism observed in SOCS6 transgenic mice (31). More evidence for a positive role for SOCS6 in cytokine signalling also comes from studies in Drosophila melanogaster where Socs44A (that is similar to SOCS6) was shown to enhance the activity of the GFR/MAPK signalling cascade, in contrast to Socs36E (similar to SOCS5) (56).

Since 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 that show high homology and similarity in binding specificity (45). Compensatory effects between SOCS cross-modulating molecules may perhaps have an effect on the phenotypes of SOCS2, -6 and -7 deficient mice (23,45,57), warranting analysis of double knock-out mice that may uncover additional physiological activities of particular SOCS proteins. It is of note that the SOCS-box is not limited to the SOCS protein family, but that at present 128 proteins harboring a SOCS-box have been described in the mammalian genome (according to Pfam database).

In summary, our findings point to the existence of a subfamily of SOCS proteins consisting of SOCS2, -6 and –7, capable of controlling SOCS protein stability. This functional cross-modulation between SOCS proteins requires the SOCS-box, probably both as inter-SOCS binding domain, and as functional recruitment motif for elongin BC-containing E3 ubiquitin ligases. This observation that several SOCS proteins not solely act as inhibitors of cytokine signalling should be taken in consideration in the evaluation of gene knock-out studies, and may be of relevance for several human pathologies.

Acknowledgments

We thank Dr. Starr for providing the pEF-Flag-I/mSOCS1, pEF-Flag-I/mCIS and pEF-Flag-I/mSOCS2 constructs, Dr. Tartaglia for the pMET7-mLR vector and Dr. Strous for the pcb6-rbGHR plasmid. The SOCS2 antibody was kindly donated by Dr. Johnston. This work was supported by grants from the Flanders Institute of Science and Technology (GBOU 010090 grant), from The Fund for Scientific Research – Flanders (FWO-V Grant N° 1.5.446.98, to J.P and D.L.) and from Ghent University (GOA 12051401).

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.

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Conclusions

Interactions between proteins are fundamental to virtually every biological process. Proteins can function as components of large, highly structured complexes that carry out specific biological roles within the cell. Examples include complex enzymatic machines such as the proteasome or the different signalosomes, but also ribosomes or enhanceosomes that interface with other macromolecules. Likewise, the basic cellular structure is maintained and controlled by the protein-based cytoskeleton. Most of these protein-protein interactions are quite stable. However, since cellular processes are context-dependent, they are subject to regulation, often by temporary protein-protein interactions.

As described in chapter 4, a broad range of both biochemical and genetic techniques have been developed to study the interactions between proteins. The in-depth characterisation of a particular protein-protein interaction or the comprehensive large-scale determination of protein-protein interactions within a particular organism requires the complementary use of different methodologies, with each tool adding unique advantages and opportunities as well as having specific drawbacks. It is thus not so surprising that the recently described large-scale TAP-based identification for protein-protein interactions in yeast showed less than 75% overlap with the datasets previously obtained by yeast two-hybrid based strategies (Gavin et al., 2006).
The 'in vitro' biochemical methodologies comprise affinity-based purification of protein complexes including peptide affinity chromatography, tandem affinity purification and co-immunoprecipitation analysis, which were all used throughout this thesis. These tools allow detection of binary interactions as well as large macromolecular protein complexes and can be applied in a variety of cell types and organisms. Combined with mass spectrometry and database searching they allow high-throughput analysis. However since cell lysis is inevitable in biochemical analysis, the normal cellular context of protein complexes may be corrupted. Moreover, given the dynamic of a cellular context, many protein-protein interactions involved in temporal intracellular processes including receptor signal transduction systems are transient and may therefore not be detectable using biochemical methodologies.

The genetic two-hybrid approaches are 'in vivo' techniques that rely on linking proteins to functional polypeptides in such a way that when these proteins interact in a living cell a detectable signal is generated. The widely used yeast two-hybrid method detects protein-protein interactions in intact yeast cells based on reconstruction of functional transcription factors. The yeast two-hybrid technique can very easily be up-scaled for high throughput analysis using either random cDNA library screening or using arrays based on complete orfeomes. However, there are some major drawbacks to the technique. Because the protein interactions occur in the nucleus the method is not suitable for membrane-anchored or large proteins. Numerous false positives occur due to the transcription-based read-out that is also located in the nucleus. Moreover, posttranslational modifications are not always correctly reproduced in yeast cells and modification-dependent protein interactions are therefore difficult to identify.

A variety of alternative two-hybrid systems in yeast mammalian cells, including the MAPPIT technique, have been developed to overcome these problems. MAPPIT, which was developed in our lab, functions in intact mammalian cells and is founded on the basic principles of type I cytokine receptor signalling (Eyckerman et al., 2001). The method relies on functional complementation of a STAT3 recruitment-deficient LR with the STAT3-recruiting domains of the gp130 chain when interaction of the fused proteins occurs. JAK2-mediated phosphorylation of the STAT3 recruitment sites then allows STAT3 docking and activation. STAT3 subsequently translocates to the nucleus and induces an easily detectable luciferase reporter gene. The MAPPIT method is versatile and adds some unique advantages to existing methods. The analytic use of MAPPIT is simple and does not require specialized machinery (Eyckerman et al., 2002). The MAPPIT technique takes benefit from the fact that STAT molecules have the intrinsic capacity to shuttle from the cytosol to the nucleus. Therefore the signal read-out in MAPPIT depends on interactions occurring in the cytosolic submembranery space and does not require translocation of bait or prey proteins to the nucleus. Interactor and effector zones are thus separated, eliminating direct interference of the bait or prey proteins with activation of the reporter gene. In addition, the fact that the read-out depends on ligand-driven receptor activation adds a supplemental level of control to monitoring interactions. Taken together, an intrinsic characteristic of MAPPIT is the low rate of false positive

results. A disadvantage of this is that because of the crucial role for STAT3 in transferring the MAPPIT signal to the reporter read-out system, bait proteins that directly lead to activation of STAT3 can not be used in MAPPIT. In addition, prey proteins associating with the cytosolic tail of the LR or with JAK2 may also cause false positive signals. However, when using two chimeric receptors with different extracellular domains and only one carrying a C-terminal bait, false positives due to LR or JAK2 association can easily be identified. Over the past years a list of such false positive interactors was worked out, with examples including the Ring finger protein 41 and the p85 subunit of PI-3K. We generated the GGS-MAPPIT variant, wherein most of the intracellular LR tail is replaced by a stretch of GGS repeats. This variation of MAPPIT eliminates false positives that are due to LR (but not JAK2) interaction. Since the MAPPIT approach is based on the cytokine-induced JAK-STAT signalling pathway in intact mammalian cells it is especially suited for, but not limited to examining protein-protein interactions that occur in signalling cascades. Both modification-independent and JAK2-mediated tyrosine phosphorylation-dependent interactions can be detected. An adapted MAPPIT variant, heteromeric MAPPIT, was developed that allows identification of modification-dependent interactions other than tyrosine phosphorylation, e.g. serine phosphorylation (Lemmens et al., 2003). In this thesis, we also demonstrated that weak, transient interactions could be detected, likely as a consequence of the inherent features of the signalling-based strategy. In further support of this, we showed the dynamic nature of the interaction of a STAT5B prey with the EpoR using electrophoretic mobility shift assays (EMSA). After recruitment to the receptor, STAT5B preys translocate to the nucleus and bind to STAT5 responsive DNA motifs (Montoye et al., 2005).

MAPPIT appears to be remarkably insensitive to sterical constraints. We have shown in this thesis that the CIS prey protein can be recruited to the Y985 and Y1077 positions in the LR in a LR-MAPPIT setup as well as to C-terminally fused EpoR domains in the classic MAPPIT system. This demonstrates that prey recruitment can occur at various positions of the chimeric bait receptor and, since both CIS association and prey-dependent STAT recruitment and activation depend on tyrosine phosphorylation, implies that the receptor-associated kinase can recognize and phosphorylate all these positions. This topological flexibility is probably due to a high degree in free rotation of the unstructured intracellular receptor tail. This is in line

with the fact that although the crystal structure of extracellular receptor domain was determined for several cytokine receptors, little is known about the structure of intracellular receptor domains (Walter, 2002). An alternative explanation could perhaps involve ligand-dependent alterations in the on-off rate of the kinase association with the receptor. The GGS-MAPPIT variant which was mentioned above, may add to the flexibility of the system.

MAPPIT experiments can be performed in a variety of mammalian cells as was demonstrated in this thesis. In many cases, this should allow MAPPIT-based examination of protein-protein interactions in the same cellular background as the signal transduction pathways are analysed. For some cell types however, endogenous STAT3 expression levels may be insufficient to generate a MAPPIT signal. In certain cell types, e.g. hematopietic cell types, this may be overcome by switching to a more abundant STAT molecule e.g. STAT5, which requires fusion of the prey proteins to the STAT5-recruiting β_c chain (Montoye et al., 2006).

As the biochemical methodologies and the yeast two-hybrid technique mentioned above, MAPPIT does not allow real time registration of protein-protein interactions, which is a unique feature of the FRET and BRET (bioluminescence resonance energy transfer) methodologies. These more complicated techniques identify interactions between proteins in intact cells based on resonance energy transfer between protein-conjugated donor and acceptor molecules.

Next to analytic applications, MAPPIT can be used as a screening tool based on reporter genes that allow selection by either selective medium or by FACS sorting (Eyckerman et al., 2002; Lievens et al., 2004). Future prospects for the MAPPIT methodology include the direct in-cell monitoring of activated phospho-STAT3 by means of InfraRed imaging with the Odyssey equipment (LI-COR). Since this eliminates the reporter amplification step, it will allow very fast read-out and opens the way to automatisation of the system. Other adaptations to the MAPPIT technique include reverse MAPPIT that permits the identification of compounds that disrupt specific protein-protein interactions and MASPIT (mammalian small molecule-protein interaction trap), a three-hybrid system for compound target identification (Eyckerman et al., 2005; Caligiuri et al., 2006).

In this thesis we focussed mainly on regulation of LR signalling. Leptin is known to act as an adipostat in energy homeostasis. It is produced in white adipose tissue and communicates the status of body fat reserves to the hypothalamus. It is also involved in a number of other often peripheral processes including immune regulation. Since alterations in normal leptin action have severe pathological implications, it is important to understand the mechanisms that regulate leptin signalling.

Until recently, the study of negative regulation of LR signalling was mainly restricted to PTP-1B and SOCS3. PTP-1B is a protein tyrosine phosphatase that predominantly targets LR signalling by dephosphorylation of JAK2 (Cheng et al., 2002; Kaszubska et al., 2002; Lund et al., 2005; Zabolotny et al., 2002). PTP-1B deficient mice are hypersensitive to leptin and protects them from high fat diet obesity, indicating that PTP-1B is a key mediator involved in leptin resistance (Elchebly et al., 1999). The precise sub-cellular location of LR signalling inhibition by this mostly endoplasmic reticulum-associated phosphatase remains to be clarified.
As part of a typical negative feedback loop, SOCS3 was identified as a potent inhibitor of LR signalling (Bjorbaek et al., 1998). Neuronal-cell specific SOCS3 deficiency or haploinsufficiency leads to leptin hypersensitivity in the hypothalamus of mice and suggests an important role for SOCS3 in central leptin regulation and resistance (Howard et al., 2004; Mori et al., 2004). SOCS3 terminates LR signalling by inhibiting JAK kinase activity via its KIR domain. It was shown to associate with the membrane proximal Y985 in the LR and to a lesser extent also with the Y1077 position (Suzuki et al., 1998; Bjorbaek et al., 2000; Eyckerman et al., 2000).

In the work presented in this thesis, we used the MAPPIT technique to introduce CIS and SOCS2 as two new interaction partners of the LR. We examined the binding properties of SOCS proteins in greater detail and investigated the functional cross-modulation between SOCS proteins.

Overall, the highly related SOCS proteins, CIS and SOCS2, show a great overlap in their interaction pattern with cytokine receptors including with the GHR and most of the tyrosines in the EpoR (Montoye et al., 2006; Uytendaele and Lemmens, in

preparation). Using both the LR-MAPPIT and GGS-MAPPIT variants we could demonstrate a differential interaction pattern of CIS and SOCS2 with tyrosines of the LR. Whereas both CIS and SOCS2 associate with the Y1077 position, only CIS interacts with the membrane proximal Y985. We also demonstrated differences in binding properties for the LR. For SOCS2 we suggested a role in regulation of STAT5 activation. Similar data were obtained in both HEK293 cells and in haematopoietic TF-1 cells. Furthermore, while SOCS2 did not associate with Y985 of the LR, we found that SOCS2 can also block interaction of CIS with this position.

Using both MAPPIT and peptide affinity chromatography we demonstrated that besides the SH2 domain, the SOCS-box of SOCS proteins can also contribute to substrate recognition. The SOCS-box of CIS is essential for interaction with EpoR and LR. Deletion of the SOCS-box or mutation of a single conserved C-terminal tyrosine completely prevented receptor interaction and resulted in abrogation of the inhibitory effect of CIS on EpoR signalling. In line with this, the SOCS-box of CIS was reported before as essential for the apoptotic effect of CIS on erythroid progenitor cells (Ketteler et al., 2003). Conversely, the SOCS-box was of no importance for interaction of CIS with MyD88, a universal adaptor protein in TLR signalling. This effect of the SOCS-box on receptor association seems to be an exclusive property of CIS since the SOCS-box was not essential for binding of the other tested SOCS - substrate interactions.

From modelling studies based on the recently determined crystal structure of the SOCS2 and elongin B/C complex one can assume that the C-terminus of CIS is also buried in the interface between the SH2 domain and the SOCS-box, suggesting that it is not directly involved in CIS interaction with the receptor or in elongin C binding (Bullock et al., 2006). Tandem affinity purification experiments confirmed that the C-terminal tyrosine of CIS is not directly involved in association with the elongin B/C complex. Similar to these observations, the C-terminus of another SOCS-box protein, the Von Hippel-Lindau protein, was also demonstrated to be involved in substrate recognition (Lewis et al., 2004). It is tempting to speculate that the mechanism by which the SOCS-box regulates receptor association of CIS may involve modification of the C-terminal tyrosine. Tyrosine phosphorylation of SOCS proteins has been described before, including phosphorylation of the corresponding C-terminal tyrosine

of SOCS3 which was found to abrogate the interaction with elongin C (Cacalano et al., 2001; Haan et al., 2003). Metabolic labelling revealed that CIS can be phosphorylated but future efforts are needed to identify the specific phosphorylation sites in the protein. It will be of interest to further clarify the underlying regulatory mechanisms.

Earlier reports described both inhibitory and stimulatory effects of SOCS2 on GH signalling in vivo and in vitro (Favre et al., 1999; Greenhalgh et al., 2002; Metcalf et al., 2000). It was suggested that SOCS2 can interfere with the negative regulatory function of other SOCS proteins (Dif et al., 2001; Favre et al., 1999; Pezet et al., 1999). We used the MAPPIT technique to demonstrate that SOCS2 can bind to members of the SOCS family, which was confirmed by co-immunoprecipitation. We suggest that SOCS2 targets these SOCS proteins for proteasome-mediated degradation and that this phenomenon depends on elongin B/C association with the SOCS-box of SOCS2. Moreover, the SOCS-box of the target protein also appeared essential. We demonstrated that SOCS2 antagonizes SOCS1 and SOCS3 in a SOCS-box-dependent manner and restores signalling via GHR, IFNγ and LR. The unexpected discovery that SOCS2 interfered with the interaction of CIS at the Y985 position of the LR, could also be explained by the direct interaction of SOCS2 with SOCS-box of CIS. Recently, others have reported that SOCS2 enhances IL-2 and IL-3 induced signalling by interfering with other SOCS proteins (Tannahill et al., 2005). We demonstrated that like SOCS2, SOCS6 and SOCS7 also interact with all members of the SOCS protein family and that SOCS6, like SOCS2, interferes with SOCS-mediated inhibition. Unlike SOCS1 and 3 that are typically induced in a rapid and transient manner upon stimulation, SOCS2 expression usually occurs late after cytokine stimulation and is more prolonged (Adams et al., 1998; Brender et al., 2001; Pezet et al., 1999; Tannahill et al., 2005). It was reported that the advanced stage of chronic myeloid leukaemia (CML) in man is associated with elevated levels of SOCS2 (Schultheis et al., 2002). It appears that SOCS over-expression in vivo can lead to certain pathologies demonstrating the significance of a tight control on SOCS protein expression levels. SOCS2 and possibly SOCS6 and SOCS7 may be involved in restoring cellular sensitivity by overcoming the inhibitory effect of other SOCS proteins.

The over-expression of SOCS6 in transgenic mice enhances both insulin sensitivity and glucose metabolism suggesting that, similar to SOCS2 in GHR signalling, SOCS6 positively regulates insulin signalling (Li et al., 2004). To further determine the precise role of SOCS6 in insulin signalling the effect of SOCS6 on downstream signalling molecules needs to be examined. Of note, although deficiency of SOCS6 or SOCS7 in mice results in relatively mild phenotypes at birth, the double knock-out mice are embryonically lethal, perhaps pointing to the loss of redundant functions of SOCS6 and SOCS7. (Krebs et al., 2002; Krebs et al., 2004; Hilton, personal communication). More detailed analysis is needed to fully understand these different phenotypes and their implications for SOCS protein function and cross-regulation.

The binding modus of SOCS-SOCS interactions will also need further examination. SOCS-SOCS interactions are currently studied in greater detail using MAPPIT and co-precipitation experiments. Mutagenesis analysis will be used to map the motifs involved in the interaction between the SOCS proteins, although full understanding of SOCS-SOCS interactions will require co-crystallization and detailed biochemical analyses, e.g. by BIAcore biosensor technology. It is clear that the kinetics of expression but also the relative expression levels and binding affinities of SOCS proteins and their target proteins will ultimately determine the signalling amplitude. Adding even more complexity to the system, we identified in this thesis a novel layer of inter-SOCS regulation, likely involved in restoring basal responsiveness. Detailed analysis of expression patterns and binding affinities of all interaction partners in a selected model cellular background will be required to fully chart the relative importance of each of these variables.

Taken together, our findings suggest a broader role for the SOCS-box of SOCS proteins. As reported before, the SOCS-box is involved in targeting associated proteins for degradation and in SOCS protein stability. In the course of this thesis project, we demonstrated that the SOCS-box can also be essential for interaction with cytokine receptors and that it plays an essential role in cross-modulation between SOCS-proteins.

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Merci

10 November 2006, het is zover!

Graag wil ik iedereen die rechtstreeks of onrechtstreeks aan de realisatie van dit doctoraat heeft bijgedragen oprecht bedanken.

Natuurlijk in de eerste plaats een dikke merci aan mijn promotor Prof. Jan Tavernier, zonder wie er van dit werk nooit sprake zou zijn geweest. Bedankt voor de kansen die ik op je labo kreeg en voor je vakkennis en je gedreven enthousiasme die zo aanstekelijk werken. Bijzondere dank ook aan Prof. Joël Vandekerckhove die mij de kans gaf deel uit te maken van zijn vakgroep Biochemie op deze prachtige locatie en die, samen met Kris Gevaert en het team, me de proteomics-faciliteiten aanbood die ik voor mijn werk nodig had. Ik dank ook Prof. Danny Broekaert voor de vele discussies van wetenschappelijke en andere aard. Hoe druk je het ook had, je vond altijd wel een minuutje voor een luisterend oor of een bemoedigend woordje. Ik wil ook de overige leden van mijn doctoraatscommissie, Prof. Cuvelier, Prof. Vandenabeele, Prof. Becker, Prof. Renauld en Prof. Leybaert bedanken voor hun kritische evaluatie van dit werk.

Wij, 'de vijf', we begonnen samen aan ons doctoraat. En eerlijk, eigenlijk wisten we van toeten noch blazen. Maar gaandeweg konden we ons bekwamen en kreeg elk zijn eigen 'specialiteit'. Op Hannes, met zijn duizend en één weetjes, kon je steeds je meest prangende of absurde vragen afvuren. Julie, altijd in voor een feestje, en Tony, meester BBQ-er, samen vormden we ons zelfverklaarde 'SOCS-team' en met spijt in het hart zie ik het nu uiteen vallen (GO SOCS!). Peter, mijn labomaatje, bij jou kon ik echt voor vanalles terecht, van gezellige wandelingetjes naar de Spar tot paniekerige telefoontjes, en je weet wel dat ik je daar bijzonder dankbaar voor ben. Hannes is ondertussen al nieuwe horizonten gaan verkennen, en Tony volgt weldra. Julie en Peter, ik hoop dat jullie nog een hele tijd op het labo zullen blijven hangen.

Een welgemeende dankjewel ook aan alle postdocs van het labo. Lennart, merci voor het doornemen van mijn schrijfsels, logistieke ondersteunig en zoveel andere dingen. Sam, ondertussen volleerd bouwvakker, en Irma, high-throughput woman, bedankt voor de praatjes in de weefselkweek, de interesse en de tips bij 't schrijven. Frank, letterlijk onze verstrooide professor, dankjewel voor alle modelletjes. Els, merci voor de leuke babbels tussendoor. Ik kijk er erg naar uit om met jou samen te werken.
Dan zijn er nog de nieuwe doctoraatsstudentjes: Joris, eigenlijk al lang niet meer zo nieuw, Leentje en Isabel. Nog een paar jaartjes en dan heb je ook zo'n boekje.

Een labo is natuurlijk niets zonder een fantastisch technisch team en dat hebben we. Annick, onze ancien, bedankt voor je gouden raad bij ontelbare grote en kleine problemen. Dominiek, constructies kennen voor jou geen geheimen meer. Merci voor al die keren dat ik met mijn lijstjes kon komen aanzetten als het werk mij weer eens boven het hoofd groeide. An-Sophie en Nele, de vrolijkste meisjes van het Rommelaere, jullie zorgen voor een nieuwe, frisse wind in het labo.

Velen hebben we zien komen en gaan. Gelukkig komen er soms ook terug. José, ons weefselkweekwonder, je keerde van onder de Franse zon terug naar 't kille België

enkel om hier in een veel te warm bureau toe te komen. Ik ben blij dat je terug bent. Sven, in een vorig leven mijn toegewijde begeleider, net terug van je Zwitsers avontuur, ik hoop dat je vaak bij ons binnenspringt.

Een pluim voor de duo's 'Johan en Serge' en 'Kristien en Dirk' die ervoor zorgden dat alles altijd gesmeerd liep op praktisch en administratief vlak, ook merci aan Peter, de computerwizard.

Ik wil jullie allemaal bedanken omdat ik echt heb genoten van deze doctoraatstijd en daar hebben jullie stuk voor stuk toe bijgedragen. Ik dank je omdat ieder op zijn manier elke dag tot een fijne dag maakte.

Natuurlijk volgt nog een speciale dankjewel voor mama en papa, mijn broer Fabien en mijn vrienden. Hoewel de meesten onder jullie zich vijf jaar lang afvroegen waar ik nu eigenlijk precies mee bezig was, bleven jullie altijd meeleven en interesse tonen of op zijn minst, maar dat is al heel wat, interesse veinzen. Als je begon te vrezen dat dat doctoraat nooit meer zou af geraken…wel, hier is het dan!

Tim, mijn liefje. Mijn allerlaatste bijzondere merci is voor jou, voor duizend en één dingen elke dag. Woorden schieten tekort als het erop aan komt uit te drukken hoeveel je voor mij betekent. Misschien kom ik wel het dichtste in de buurt met één van mijn favoriete zinnetjes uit een lievelingsfilm van jou: 'The sun rises and sets with you.'

– Great things come to those who wait (EV) –

Merci,

Delphine