Review: negative regulation of leptin receptor signalling

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

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

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

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

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

JAK-STAT SIGNALLING

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

JAK2 is constitutively associated with the membrane proximal box1 in the cytoplasmatic tail of the LR [10, 11]. The intracellular part of the receptor also carries three conserved tyrosines at positions Y985, Y1077 and Y1138 (murine numbering). The membrane distal tyrosine is embedded in 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, indicative of the involvement of other signal transducers [15]. Leptin-induced activation of STAT1 and STAT5B, in addition to STAT3, was shown in COS cells and in HIT-T15 cells [16, 17]. In the latter cell line, STAT1 was activated *via* Y1138 while STAT5B activation occurred *via* both Y1138 and Y1077 [17].

Next to JAK-STAT signalling, leptin also activates other pathways. A number of adaptor molecules can associate with the receptor and link to several signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway (see below) and the phosphoinositol 3-kinase

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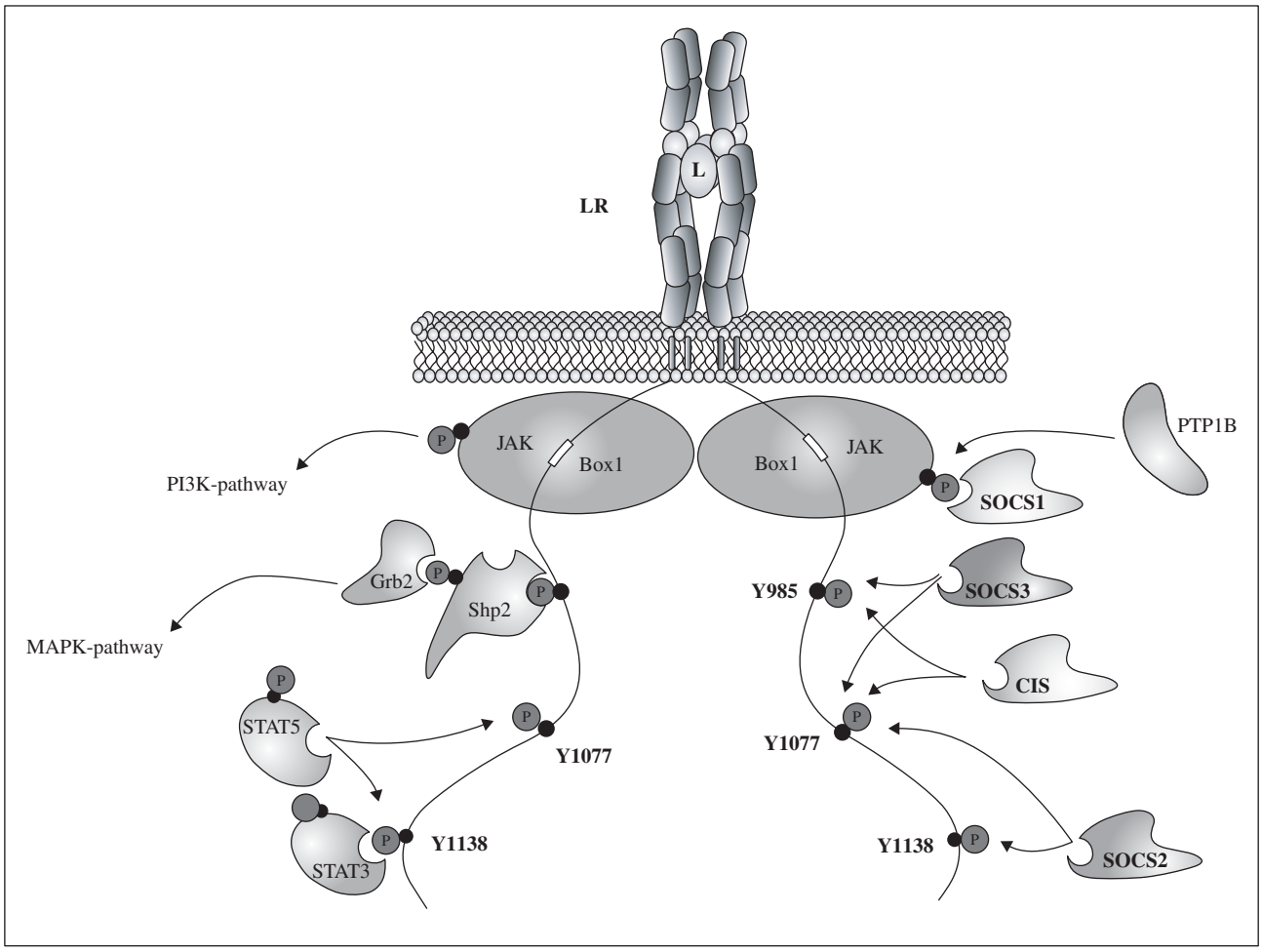

Figure 1
Schematic representation of LR signalling and negative regulation. For abbreviations, refer to the main text.

(PI3K) pathway. In the latter, the JAK2-interacting protein, SH2-B, mediates binding of IRS (insulin receptor substrate) proteins that function as adaptors for PI3K [18, 19]. PI3K transforms 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].

MODULATION OF FUNCTIONAL RECEPTOR EXPRESSION

Obviously, receptor internalisation is an effective mechanism for rapidly turning off cytokine signalling. Upon ligand binding, cytokine receptors can be 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 are found in intracellular pools [21]. This distribution of the LR may be explained by its tendency to constitutive endocytosis resulting in short-lived membrane expression. In addition, some of the newly-synthesized LRs are retained intracellularly based on a retention signal in the transmembrane domain [22]. Whether external stimuli modulate this LR localisation throughout the cell and in this way regulate leptin sensitivity remains to be determined.

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

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

A soluble form of the LR associates with circulating leptin [28]. Secreted cytokine receptors can protect their ligands

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

PHOSPHATASES

SH2 domain-containing phosphatase-2 (SHP-2) is a constitutively expressed protein tyrosine phosphatase known to be involved in the dephosphorylation of the JAKs. It carries two tandem SH2 domains followed by a tyrosine phosphatase catalytic domain and associates directly with the LR at position Y985 [37]. The exact role of SHP-2 in LR signalling has been a long standing matter of debate. Despite its initial identification as an inhibitor of LR signalling (see below), it also appeared as a strong activator of the MAPK pathway. ERK activation occurs predominantly *via* SHP-2 recruitment 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 have also attributed an inhibitory role to the SHP-2 phosphatase in LR signalling. Mutation of the Y986 position in the human LR led to augmented STAT3 signalling, and inhibitory properties associated with this position were ascribed to the negative regulatory function of SHP-2 [42]. However, suppressor of cytokine signalling 3 (SOCS3), identified as a strong inhibitor of LR signalling (see below), was found to interact with the corresponding Y985 position in the murine LR [43-45]. SOCS3 is part of the SOCS family and its inhibitory mechanism is discussed below. SHP-2 and SOCS3 have very similar binding specificities, and overlapping binding sites were also observed for the gp130 chain [46-49]. Thus, the negative regulation associated with the membrane proximal tyrosine position is partly attributed to SOCS3. However, SHP-2-mediated dephosphorylation of JAK2 was demonstrated *in vitro* [37]. Recently, forebrain-specific SHP-2-deficient mice revealed that SHP-2 moderately down-modulates JAK2 and STAT3 activation *in vivo* [50]. Although SHP-2 has a modest role in terminating leptin signal transduction, its dominant induction of the ERK pathway makes it overall an enhancer of leptin signalling, whereby it may function as a switch towards MAPK signalling.

Protein tyrosine phosphatase 1B (PTP1B) is a crucial protein tyrosine phosphatase implicated in the negative regulation of leptin receptor signalling. PTP1B deficiency results in hypersensitivity to insulin and leptin in mice, and leads to protection from high fat diet obesity [51]. PTP1B harbours two phosphotyrosine binding pockets in its catalytic domain that determine its intrinsic specificity. A consensus substrate recognition motif was found in the kinase activation loop of the insulin receptor and in JAK2 [52-54]. Both *in vivo* and *in vitro* data demonstrate that PTP1B targets LR signalling predominantly by dephosphorylating JAK2 [55-58]. PTP1B is a negative mediator of both the JAK-STAT and MAPK pathway in leptin receptor signalling. PTP1B-mediated hypophosphorylation of JAK2 in a mouse hypothalamic neuronal cell line abrogated the leptin-dependent induction of the STAT3 and MAPK inducible SOCS3 and c-fos genes, respectively [56]. Recently, leptin induced PTP1B was observed in liver, raising the possibility that PTP1B may also function in a negative feedback loop [59]. Diet-induced obesity is associated with increased hepatic PTP1B levels. Aberrant PTP1B activity is implicated in leptin resistance and PTP1B is currently being investigated as a drug target in obesity [60-63].

PTP1B is localized predominantly on the ER (endoplasmic reticulum) *via* its C-terminal hydrophobic targeting sequence [64]. How PTP1B acts on its substrates remains unclear. It was demonstrated that the platelet-derived growth factor (PDGF) receptor becomes dephosphorylized by PTP1B at the ER after internalization [65]. Recently, direct interaction of PTP1B with the insulin receptor was observed in a perinuclear endosome compartment [66]. On the other hand, it has been demonstrated that internalisation of the insulin receptor is not essential for interaction with PTP1B and subsequent dephosphorylation [67]. In line with this, proteolytic cleavage of PTP1B can lead to the relocalization of the catalytic domain of PTP1B to the cytosol [68].

The ubiquitously expressed phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumour suppressor protein and its mutation is linked with several human cancer types [69]. It belongs to the family of protein tyrosine phosphatases but also possesses lipid phosphatase activity. PTEN suppresses the PI3K pathway by hydrolyzing the secondary messenger PIP^3 back to PIP^2. [70]. It was demonstrated that hypothalamic PI3K is involved in leptin-induced reduction in food intake [19]. Surprisingly, specific disruption of PTEN restricted to the hypothalamic neurons expressing the anorexigenic proopiomelanocortin (POMC) neuropeptide results in an obese phenotype associated with leptin resistance [71].

SUPPRESSORS OF CYTOKINE SIGNALLING

The family of SOCS proteins consists of 8 members: cytokine inducible SH2 protein (CIS) and SOCS1 through SOCS7. SOCS proteins have a characteristic domain

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 kinase inhibitory region (KIR) 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.

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]. A role for leptin has also been implicated in the expression of SOCS1 and, to a lesser extent of SOCS2 [74, 75].

SOCS3 was identified as a potent inhibitor of LR signalling [43]. Its STAT3-mediated expression is induced in the hypothalamus and liver after peripheral leptin 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 show SOCS3 up as a key mediator of negative regulation of leptin signalling and suggest a prominent role in leptin resistance.

Only SOCS1 and 3 carry a KIR domain in their N-terminal region involved in direct inhibition of the JAK kinase activity. They both inhibit leptin receptor signalling, using a slightly different mechanism. SOCS1 directly interacts with the kinase domain of JAK2 by targeting the 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].

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

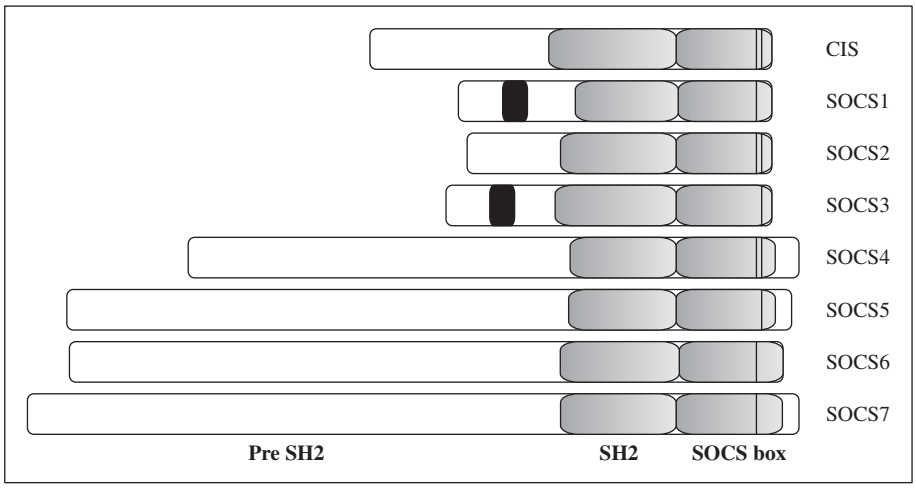

Figure 2
Schematic overview of SOCS protein structure. The KIR domain is indicated with a black box, the C-terminal, conserved tyrosines are represented by a black line.

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]

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

observe any inhibitory effect on STAT3-mediated LR signalling by either CIS or SOCS2 [73, 74]. Instead, we suggest an inhibitory role in leptin-induced STAT5 signalling through interference with STAT5a recruitment to the Y1077 tyrosine motif in a MAPPIT based experiment [74]. Supporting this notion, SOCS2 binding completely overlaps with STAT5 association at the LR. CIS and SOCS2 may be implicated in preventing recruitment of downstream signalling moieties to the LR. Both SOCS2 knockouts and CIS 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, in part, be explained by its effect on leptin signalling in T-cells. More detailed analysis in cell-type specific expression and function will be needed to elucidate the specific roles of SOCS proteins in leptin signalling. Possibly, different physiological functions of leptin may be under the control of different SOCS proteins.

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

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

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

CONCLUSION

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

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