Identification of the Y985 and Y1077 motifs as SOCS3 recruitment sites in the murine leptin receptor

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Abstract The leptin system provides a link between adipose mass and the central nervous system. The appetite suppressing effects of leptin are impaired in most obese patients and some mutant mice strains. Herein we describe how suppressor of cytokine signalling 3 (SOCS3), a potential mediator of this leptin resistance is recruited into the activated murine leptin receptor complex. Using a functional assay based on inhibition of leptin mediated reporter induction, and using phosphopeptide affinity chromatography we show binding of SOCS3 to the highly conserved phosphorylated Tyr-985 and Tyr-1077 motifs within the mouse leptin receptor.

Key words: Leptin receptor; Signal transduction; Suppressor Of cytokine signalling

1. Introduction

Leptin, an adipocyte derived hormone, delivers its appetite suppressing signals by passing the blood-brain barrier and by binding to the specific signalling form of its receptor in certain nuclei of the hypothalamus. In this way it constitutes a feedback mechanism regulating adipose tissue mass. Mutations within the leptin system result in a marked obese phenotype and impaired endocrinological functioning [1,2]. Although the long signalling form of the leptin receptor shows high expression levels in these hypothalamic nuclei, it is also expressed in several peripheral tissues including lung, liver, lymph nodes and gonads [3,4]. This leads to the involvement of leptin in several peripheral functions, making it a typical pleiotropic cytokine.

Until today the therapeutic use of leptin as a weight reducing agent remains limited [5]. It is observed that in most obese people a strong correlation exists between adipose mass and leptin levels, a phenomenon often explained by leptin resistance [6]. A number of possible explanations for this resistance have been suggested: a saturable transport through the blood-brain barrier resulting in a limited leptin activity in the hypothalamus [7,8], cross-talk with the glucocorticoid system [9], or defects at the leptin receptor level, such as elevated expression of the signalling inhibitor SOCS3 [10].

Being a member of the type 1 cytokine receptor family, the leptin receptor is activated by cross-phosphorylation of associated JAK kinases, most likely JAK2 and/or JAK1 [11,12]. Activation of the leptin receptor leads to recruitment of signalling molecules containing phosphotyrosine binding SH2 modules. Signal transducers and activators of transcription (STAT) molecules [13,14] and the receptor associated SH2 containing phosphatase SHP-2 [15,16] are both recruited in the activated leptin receptor complex. Leptin mediated activation of mitogen activated protein kinases (MAPK) and insulin receptor substrate 1 (IRS-1) has been shown in various cell systems [11,12,17,18].

Leptin can also strongly and rapidly induce the production of the signal transduction inhibitor suppressor of cytokine signalling 3 (SOCS3) in various cell types and in vivo [10,19,20]. SOCS3 is a member of an expanding family of SH2 containing proteins which are typically built up of a pre-SH2 domain, a central SH2 domain, and a highly conserved SOCS box sequence. This last motif is also found in a number of other signalling molecules [21] and seems to be connected with proteasome function [22]. The observation that the leptin resistant A^y/a mutant mice strain shows elevated SOCS3 levels makes this protein a possible mediator of leptin resistance [10].

Recently it has been shown that a tyrosine recruitment site within gp130, the signalling component of the IL-6 complex, is required for binding and thus for the inhibitory activity of SOCS3 [23,24]. This is in contrast to SOCS1, which binds directly to JAK kinases and directly inhibits their kinase activity [25]. Similar results have been obtained for the insulin receptor and the erythropoietin receptor [26,27].

In previous studies we have shown that leptin induces two gene sets in the PC12 rat pheochromocytoma cell line stably expressing the mouse leptin receptor. Many of these genes appear to be regulated in vivo [20]. Using a mutational approach we have also shown that in the mouse leptin receptor residue Y985 is involved in a negative feedback signal, and furthermore that this effect is more pronounced when mutated in concert when another tyrosine, Y1077 [28]. In this report, we analyze the role of both residues in SOCS3 recruitment.

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Abbreviations: LR, leptin receptor; rPAP1, rat pancreatitis associated protein 1; SHP, SH2 containing phosphatase; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription

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2. Materials and methods

2.1. Antibodies, growth factors and peptides

Monoclonal anti-FLAG antibody M2 was obtained from Sigma. Mouse recombinant leptin was purchased from R and D Systems. Peptides were synthesized using standard Fmoc amino acid solid phase chemistry on an Applied Biosystems Model 431A peptide synthesizer. Biotin was esterified manually following similar procedures as on the synthesizer, and added to the reaction mixture. Incorporation of phosphotyrosine was realized by using a protected Fmoc tyrosine phosphobenzylester (Novabiochem). After purification, mass of the peptides was confirmed by mass spectrometry. Sequences of the peptides used were biotin-QRQPSVK(p)Y_{985}ATLVSNDK and biotin-NHREKSVC(p)Y_{1077}LGVTSVNR.

2.2. Vectors

The pMET7-LR long isoform was kindly provided by L. Tartaglia. Generation of the leptin receptor mutants was described elsewhere [28].

Rat SOCS3 cDNA was amplified using 5′-GAAGATCTGTGCGCCATGGTCACCCACAGCAAGTT and 5′-GCTCTAGATTTTGCTCCTTAAAGTGGAGCATCATA as forward and reverse primer respectively, and using mRNA from leptin stimulated PC12 cells as template. cDNA was prepared using a standard RT procedure with superscript reverse transcriptase (LifeTechnologies). Amplification was realized using Pfu polymerase (Stratagene). The SOCS3 fragment was reamplified using forward primer 5′-GCGAGATCTCAGAATTCGTCACCCACAGCAAGTTTCC and the reverse primer described above, which allows *Bgl*II-*Xba*I based cloning in a pMET7 variant containing an N-terminal FLAG tag sequence (MDYKDDDDK). The pUT651 construct expressing β-galactosidase was obtained from Eurogentec. Generation of the pGL3-rPAP1-luci construct was described before [28]. The full-length rPAP1 promoter fragment was excised using partial digestion with *Kpn*I and *Xho*I and ligated into the *Kpn*I-*Xho*I digested pXP2d2 vector (gift from Prof. S. Nordeen), resulting in the leptin responsive pXP2d2-rPAP1-luci reporter construct. The pXP2d2 vector is a derivative of pXP2 that lacks potential cryptic activator protein 1 sites [29]. All constructs were verified by restriction and sequence analysis.

2.3. Cell lines and transfection procedures

Culture conditions and transient transfection procedures for PC12 and generation of the PC12LR8 cell line were as previously described [28,20].

HEK293T cells were maintained in a 10% CO_2 humidified atmosphere at 37°C, and were grown using DMEM with 4500 mg/l glucose, 10% fetal bovine serum and 50 μg/ml gentamicin (all from LifeTechnologies). Typically, 4×10^5 cells were seeded the day before transfection in a 6-well plate and transfected overnight with approximately 2 μg plasmid DNA using a standard calcium phosphate precipitation procedure. One day after transfection, cells were resuspended with cell dissociation agent (LifeTechnologies), seeded in a black well plate (Costar), and stimulated overnight with 100 ng/ml leptin, or left unstimulated.

2.4. Reporter assays and Northern blot

Luciferase assays, binding assays using leptin-SEAP, and Northern blot hybridizations with SOCS3 and actin probes were described previously [28]. β-Galactosidase activity was measured using the Galacto-Star™ chemiluminescent detection kit (Tropix) and a Topcount Chemiluminescence Counter (Packard).

2.5. Western blot analysis and phosphopeptide affinity chromatography

Approximately 10^6 HEK293T cells were lysed in 150 μl 2× loading buffer. After sonication, 30 μl was loaded on a 10% polyacrylamide gel. After overnight blotting, FLAG-tagged SOCS3 was revealed using a 1/2500 dilution of anti-FLAG antibody. Blotting efficiency was checked using PonceauS staining (Sigma). For phosphopeptide affinity chromatography, approximately 3×10^7 HEK293T cells transiently transfected as indicated 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; 1 mM $NaVO_4$; 5 mM NaF; 20% glycerol; Complete™ Protease Inhibitor Cocktail [Roche]). Precipitated material was cleared by 5 min centrifugation at $10\,000\times g$. To eliminate a-specific interactions, supernatants were brought on a precolumn containing

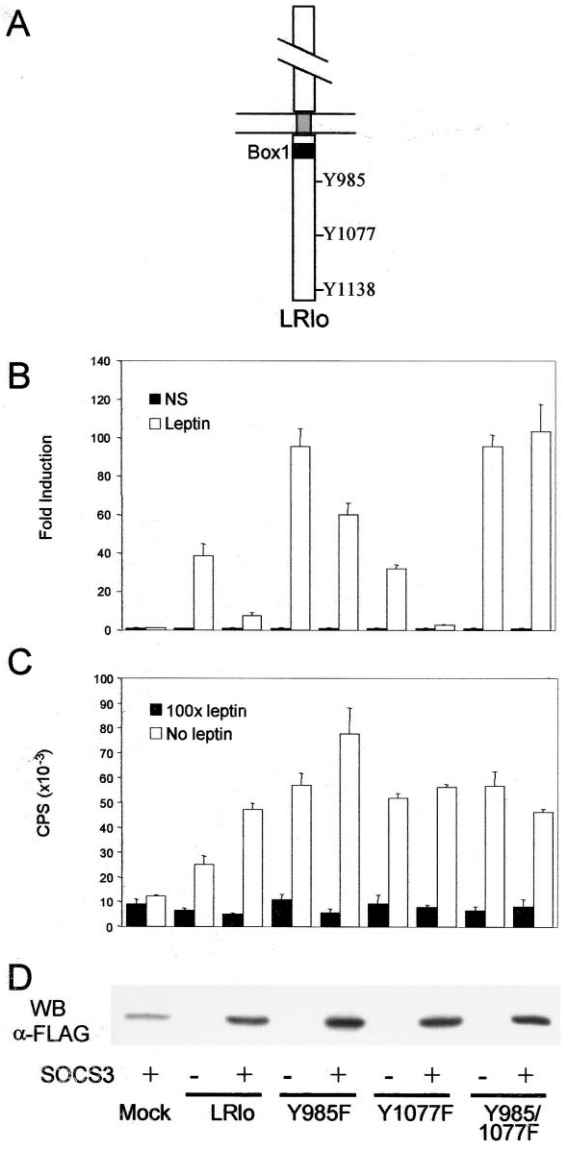

Fig. 1. SOCS3 mediated inhibition of leptin signalling is critically dependent on the presence of either Y985 or Y1077 in the murine leptin receptor. A: Schematic representation of the long isoform of the murine leptin receptor (LRlo), showing extracellular (EC), transmembrane (TM) and intracellular (IC) parts. The box1 motif and the three tyrosines involved in signal transduction are also shown. B: Hek293T cells were transiently co-transfected with plasmids encoding different leptin receptor variants, SOCS3 or empty vector, together with the pXP2d2-rPAP1-luci reporter construct. The transfected cells were either stimulated for 24 h with leptin or were left untreated. Luciferase measurements were performed in triplicate and were normalized by co-transfection with the pUT651 β-galactosidase construct and a β-galactosidase activity assay. Results are shown in fold induction and are representative for three separate transfection experiments. Significance for the results obtained for LRlo, Y985F and Y1077F was $P<0.001$ when the three separate experiments were pooled and analyzed by two-way ANOVA. C: LR expression levels were measured on transfected cells by incubation for 90 min with leptin-SEAP fusion protein with or without excess leptin (100×). Bars show mean values and S.D. values of triplicate measurements. D: Western blot analysis of SOCS3 expression. The FLAG-tagged SOCS3 protein was revealed in lysates of transfected cells using anti-FLAG antibody. CPS, absolute luminescence counts per second; LRlo, leptin receptor long isoform.

Sepharose 4B beads, prior to phosphopeptide affinity chromatography on streptavidin coupled agarose beads (Sigma). Peptide concentrations were determined by a colorimetric assay using alkaline hydrolysis and a ninhydrin reagent. 50 μl of streptavidin–agarose slurry was incubated with 5 nmol of peptide for each reaction. Cleared lysate was incubated for 2 h at 4°C under slow stirring. After incubation, beads were washed four times with lysis buffer and resuspended in 2× loading buffer.

3. Results and discussion

The observation that most human obese patients show elevated levels of leptin suggests the existence of a so-called leptin resistance [6]. SOCS3, a potential mediator of this resistance [10] is a member of a family of SH2 domain containing proteins mostly involved in negative regulation of signal transduction pathways [21]. Through representational difference analysis, a PCR based differential expression screening, we were able to identify several leptin induced transcripts, including SOCS3, in PC12 cells expressing the mouse leptin receptor. In order to check the inhibitory role of SOCS3, we generated an expression vector containing rat SOCS3. Transient expression of SOCS3 in HEK293T cells together with the leptin responsive pXP2d2-rPAP1-luci construct leads to marked inhibition of reporter induction (Fig. 1B: results for LRlo). The rPAP1 promoter is derived from the rat pancreatitis associated protein 1 gene and shows strong induction upon treatment with leptin ([20] and Broekaert et al., in preparation). Similar findings were obtained in PC12LR8 cells [20], a PC12 clone that stably expresses the leptin receptor (data not shown).

Recently it has been shown that SOCS3 can mediate its inhibitory activity by binding to activated receptors although the exact mechanism of inhibition remains unclear. Binding of SOCS3 was demonstrated for the gp130 signalling component of the IL-6 complex [23,24], the erythropoietin receptor [27] and the insulin receptor [26]. Based on the functional similarities between the leptin receptor and the gp130 chain [13], we tested the leptin receptor for interaction with SOCS3. The murine leptin receptor contains three tyrosine residues within its cytoplasmic domain (Fig. 1A). Y1138 is situated within a box3 or STAT3 recruitment motif and is involved in leptin mediated gene induction [30]. We previously showed a critical role in negative feedback for the Y985 and to a lesser extent also for the Y1077 residue [28]. Leptin receptor variants containing Y to F mutations at positions Y985, Y1077, or at both sites, were tested in a functional assay based on rPAP1 induction. Signalling via the leptin receptor variants was analyzed upon transient transfection in HEK293T cells with the pXP2d2-rPAP1-luci and the FLAG-tagged pMET7-fSOCS3 expression construct. Luciferase activity data were normalized by co-transfection with pUT651 and a β-galactosidase activity assay (Fig. 1B). Expression of leptin receptor variants and SOCS3 was confirmed respectively by a binding assay using a leptin-SEAP fusion protein (Fig. 1C) and Western blot analysis using an anti-FLAG antibody (Fig. 1D). The results indicated a strong inhibitory activity of SOCS3 (80–90% inhibition) when either the wild-type or the mutant Y1077F leptin receptor was expressed, and a moderate inhibition (30–50% inhibition) upon expression of a Y985F receptor variant. In case of the double Y985/1077F mutant no inhibition was observed.

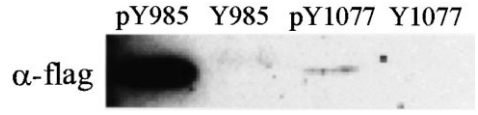

Fig. 2. SOCS3 binding to peptides matching the murine leptin receptor Y985 and Y1077 sites is phosphorylation dependent. Lysates of Hek293T cells overexpressing FLAG-tagged SOCS3 protein were incubated with peptides matching leptin receptor Y985 and Y1077 sites, either phosphorylated or not. Specific binding of SOCS3 was revealed by immunoblotting using an anti-FLAG antibody.

The interaction sites of SOCS3 with the leptin receptor were confirmed using a biochemical approach. FLAG-tagged SOCS3 was expressed upon transient transfection of pMET7-fSOCS3 in HEK293T cells, and was analyzed for binding to (phospho)-tyrosine containing peptides matching the two motifs within the leptin receptor. Lysates of approximately 3×10^7 transfected cells were incubated with biotinylated peptides encompassing residue Y985 or Y1077 in the leptin receptor. Western blot analysis using an anti-FLAG antibody showed clear and specific binding of SOCS3 to the phosphorylated Y985 peptide, while non-phosphorylated peptide did not bind any SOCS3 protein. SOCS3 also binds specifically to the phosphorylated Y1077 peptide but apparently with a much lower affinity confirming its accessory role in SOCS3 mediated inhibition (Fig. 2).

To assess the effect of Y to F mutations within the leptin receptor on SOCS3 expression itself, Northern blot analysis was performed on lysates from transiently transfected PC12 cells. The neuronal differentiation pattern and the fact that

Table 1
Conservation of the Y985 and Y1077 motifs within the leptin receptor

Accession number	Species	Y985										Y1077												
AAC52705	Mus musculus	V	K	Y	A	T	L	V	**S**	N	D	K	V	C	Y	L	G	V	T	**S**	V	-	N	R	R	E
AAF89300	Rattus norvegicus	V	K	Y	A	T	L	V	**S**	N	V	K	V	Y	Y	L	G	V	T	**S**	S	G	N	K	R	E
AAA93015	Homo sapiens	V	K	Y	A	T	L	I	**S**	N	S	K	I	Y	Y	L	G	V	T	**S**	I	-	K	R	R	E
AAF34683	Macaca mulatta	V	K	Y	A	T	L	I	**S**	N	S	K	I	Y	Y	L	G	V	T	**S**	I	-	K	K	R	E
AAC48708	Ovis aries	V	K	Y	A	T	L	V	G	**S**	S	K	V	Y	Y	L	G	V	T	**S**	I	-	K	K	R	E
AAB88825	Sus scrofa	I	K	Y	A	T	L	L	**S**	S	P	K	V	Y	Y	L	G	V	T	**S**	I	-	K	K	R	
AAB40624	Bos taurus	V	K	Y	A	T	L	L	**S**	N	S	K	V	Y	Y	L	G	V	T	**S**	I					
BAA94292	Gallus gallus	I	K	Y	A	T	V	I	**S**	N	S	R	L	H	Y	L	G	I	T	**S**	L	-	G	K	R	E
AAD31284	Equus caballus	V	K	Y	A	T	L	L	G	**S**	S	K														
		*			*		*		*			*	*			*		*		*				*		

The conserved tyrosine is shown boxed; a conserved serine at position +5 (or +6) found in both motifs is shown in bold. Asterisks below indicate conserved amino acid residues shared by both motifs. Dashes in the Y1077 motif were introduced for optimal alignment.

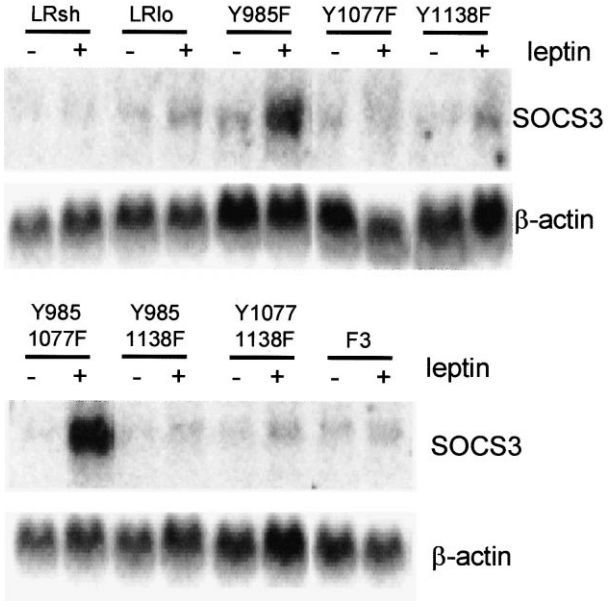

Fig. 3. Y985F and Y985/1077F, but not Y1077F mutations in the leptin receptor lead to enhanced induction of SOCS3 mRNA. PC12 cells were transiently transfected with plasmids encoding different leptin receptor variants and were stimulated with leptin for 24 h. Northern blot analysis was performed on lysates using a rat SOCS3 probe. A β-actin probe was used for mRNA quantitation. LR, leptin receptor; sh, short isoform; lo, long isoform; F3, all cytoplasmic tyrosines mutated to phenylalanine.

several of the leptin induced genes in these cells also show in vivo modulation [20], makes this cell line a valuable model system to study leptin signalling. 48 h after transfection, cells were stimulated for 24 h with leptin (100 ng/ml) or were left untreated (Fig. 3). Previously obtained results indicate that SOCS3 transcription is rapidly induced in PC12 cells with an optimum around 30 min, but that weak SOCS3 expression persists until later time points [20]. Results shown in Fig. 3 imply that during this late induction phase, mutation of Y985 results in a strong up-regulation of SOCS3 transcription. This occurs only in conjunction with Y1138, a STAT3 activation site, indicating that SOCS3 expression is STAT dependent, as has been shown before for signalling via the IL-6 and leukemia inhibitory factor receptors [31]. Absence of the Y1077 site does not lead to altered SOCS3 mRNA expression, but lack of both sites results in a further increased induction level above what is observed for the Y985 mutant alone. These results are in line with our previous results for metallothionein II mRNA regulation and rPAP1 induction [28]. A possible explanation for the elevated SOCS3 expression level could be the loss of negative feedback via either SOCS3 itself, or alternatively via the SH2 containing phosphatase SHP-2 [15,16].

Taken together, these findings suggest that both tyrosines are involved in SOCS3 recruitment to the activated leptin receptor complex although binding to the Y1077 position apparently occurs at significantly lower efficiency suggesting an accessory role for this site. This observation could explain why, at late time points post stimulation, the single Y1077 mutant does not lead to detectable differences on induction of metallothionein II and SOCS3 genes when compared to the wild-type receptor, whereas a more pronounced induction is observed when the double Y985/1077F mutant is compared to the Y985F receptor variant (see Fig. 3, and also [28]). In line with these findings, recruitment of SOCS3 at the single phosphorylated Y1077 motif shows a moderate inhibition of leptin signalling (Fig. 1). Perhaps this accessory role of the Y1077 motif is only functional when expression levels of SOCS3 are highly elevated, suggesting different threshold levels for leptin receptor signalling may exist. Although several groups were not able to show leptin dependent phosphorylation of the Y1077 site in the leptin receptor using anti-phosphotyrosine antibodies [12,16], our results indicate a functional phosphorylation dependent role for this site in leptin signalling. This could perhaps be explained by the fact that the anti-phosphotyrosine antibodies used do not recognize this site, or that only marginal phosphorylation occurs. This latter possibility could be mediated by the presence of a signalling protein that binds (close to) the Y1077 site, preventing its recognition by kinases.

Functionality of the Y1077 motif is also strongly suggested by the cross-species conservation of this sequence. In fact, Y1077 is part of a highly conserved motif extending 10 amino acids downstream of the tyrosine residue (Table 1). This motif is also related to the Y985 flanking sequence, with conserved hydrophobic residues at positions −2, +1 and +3, and a conserved basic residue at +8. Intriguingly, besides this conserved motif encompassing a canonical SHP-2 binding site [32], a serine residue also appears to be conserved within both motifs at position Y+5, suggesting a shared function. This serine is shifted to position +6 in the horse and ovine LR Y985 motif due to an extra glycine at position +5. Perhaps, phosphorylation at this serine residue may modulate the interaction with signalling regulators. Taken together, the pattern of conservation of the Y985 and Y1077 motifs provides a molecular basis for the binding of both shared, such as SOCS3, and unique signalling components. The very pronounced conservation of the Y1077 motif also underscores its functional importance in leptin signalling.

It has been demonstrated that the SH2 containing phosphatase SHP-2 is also recruited to the phosphorylated Y985 motif receptor [15,16]. It therefore appears that at least two signal modulating proteins can bind to the phosphorylated Y985 site, suggesting a complex control of leptin receptor (in)activation. Insights in the relative binding affinities and spatiotemporal expression patterns of these signalling modulators may help understand leptin resistance.

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