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Negative Regulation of Growth Hormone (GH) Signaling

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Real success is finding your lifework in the work that you love

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In loving memory
of my father and
my brother

To my Family

ABSTRACT

Growth Hormone (GH) regulates postnatal longitudinal growth. It also has several other functions. It decreases cholesterol and lipoproteins levels, reduces fat tissue and increases lean body mass. GH actions are mediated by the JAK2/STAT5 signaling pathway. The manner whereby this pathway is activated has been extensively studied. There are, however, gaps in our knowledge how the pathway is silenced or negatively regulated. Down regulation of GH signaling is related to several interconnected mechanisms mainly involving the internalization of GH receptor (GHR) and the action of negative regulators such as tyrosine phosphatases and suppressors of cytokine signaling (SOCS). SOCS proteins have recently received attention as important regulators of the JAK/STAT pathway. In relation to GH, it is interesting to note that mice lacking SOCS2 ($SOCS2^{-/-}$) are 40% larger that their littermates. While this evidence suggests that SOCS2 controls GH and/or IGF-I signaling, the exact mechanism behind this action has not been defined yet. One hypothesis is that GHR turnover is critical and that SOCS proteins take part in this process by regulating protein breakdown through ubiquitination.

The aim of this thesis was to characterize pathways that regulate GH sensitivity in liver and to evaluate the mechanism of action of SOCS proteins, particularly SOCS2, as negative regulators for GH signaling pathway.

We demonstrated that the duration of GH-activated JAK2/STAT5 can be prolonged by other signaling pathways, such as the UPR (Unfolded Protein Response) and those regulating the actin cytoskeleton. These two pathways target different steps in the down regulatory pathway involving GHR ubiquitination and endocytosis. Induction of UPR decreases the degree of GHR ubiquitination while actin cytoskeleton disruption results in accumulation of ubiquitinated GHR. Mice lacking SOCS2 ($SOCS2^{-/-}$) were analyzed using transcript profiling and other means to evaluate their phenotype. We demonstrated that $SOCS2^{-/-}$ mice have limited similarity with GH-overexpression models. These mice are therefore not an exact mimic of GH overproduction and instead they represent “a state of their own” in terms of gigantic phenotypes. Crossing $SOCS2^{-/-}$ mice with GH deficient dwarf mice resulted in a dwarf phenotype, indicating that the gigantism of $SOCS2^{-/-}$ mice depends on endogenous GH secretion. When the two dwarf models were compared, lack of SOCS2 resulted in hypersensitivity to GH treatment, as was demonstrated by increased growth rate and exaggerated responsiveness of hepatic GH-regulated genes. The mechanism of SOCS2 action involves its binding to GHR through the tyrosines 487 and 595 and its association to Elongin BC, probably forming an ubiquitin ligase complex that could mediate GHR ubiquitination and degradation. In conclusion, our studies indicate that GH signaling down regulation is a tightly regulated process, involving several steps. It is modulated by UPR, actin cytoskeleton network, SOCS proteins (particularly SOCS2), the rate of GHR ubiquitination and might include the action of some still unidentified proteins that could contribute to the negative action of SOCS2.

LIST OF PUBLICATIONS

This thesis is based in the following papers, which will be referred to in the text by their Roman numbers:

I. Amilcar Flores-Morales, Leandro Fernández, **Elizabeth Rico-Bautista**, Adriana Umana, Ciro Negrín, Jian-Guo Zhang and Gunnar Norstedt.

Endoplasmic Reticulum Stress Prolongs GH-Induced Janus Kinase (JAK2)/Signal Transducer and Activator of Transcription (STAT5) Signaling Pathway. *Molecular Endocrinology*. 2001. 15 (9): 1471-1483

II. **Elizabeth Rico-Bautista**, Ciro Negrín-Martínez, Javier Novoa-Mogollón, Leandro Fernández-Pérez and Amilcar Flores-Morales.

Downregulation of the growth hormone-induced Janus Kinase 2/signal transducer and activator of transcription 5 signaling pathway requires an intact actin cytoskeleton. *Experimental Cell Research*. 2004. 294 (1): 269-280

III. **Elizabeth Rico-Bautista**, Christopher J. Greenhalgh, Petra Tollet-Egnell, Douglas J. Hilton, Warren S. Alexander, Gunnar Norstedt and Amilcar Flores-Morales.

SOCS2 deficiency induces molecular and metabolic changes that partially overlap with GH-dependent effects. *In press Molecular Endocrinology. March, 2005. Online November 24, 2004.*

IV. Christopher J. Greenhalgh, **Elizabeth Rico-Bautista,** Mattias Lorentzon, Anne L Thaus, Phillip O. Morgan, Tracy A. Willson, Panagiota Zervoudakis, Donald Metcalf, Ian Street, Nicos A. Nicola, Andrew D. Nash, Louis J. Fabri, Gunnar Norstedt, Claes Ohlsson, Amilcar Flores-Morales, Warren S. Alexander, and Douglas J. Hilton.

Suppressor of Cytokine Signaling 2 negatively regulates growth hormone action in vitro and in vivo. *In press Journal of Clinical Investigation*.

Related Paper.

1. Nina Ståhlberg*, **Elizabeth Rico-Bautista***, Rachel M. Fisher, Xuxia Wu, Louisa Cheung, Amilcar Flores-Morales, Gunnel Tybring, Gunnar Norstedt and Petra Tollet-Egnell.

Female-Predominant Expression of Fatty Acid Translocase/CD36 in Rat and Human Liver. *Endocrinology*. 2004 Apr; 145(4):1972-9. *Contributed equally to this study.

ABBREVIATIONS

(-/-)	Homozygous gene-deficiency
ALS	Acid Labile Subunit
BRL	Buffalo Rat Liver
Cyto D	Cytochalasin D
DAG	Diacylglycerol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase enzyme
ER	Endoplasmic Reticulum
FAK	Focal Adhesion Kinase
GEMSA	Gel Electrophoretic Mobility Shift Assay
GH	Growth Hormone
GHBP	Growth Hormone Binding Protein
GHR	Growth Hormone Receptor
GHRH	Growth Hormone Releasing Hormone
GHRHR	Growth Hormone Releasing Hormone Receptor
$Ghrhr^{lit/lit}$	Growth Hormone Releasing Hormone Receptor (Point Mutation)
HBP	Helix Bundle Peptide
HNF	Hepatic Nuclear Factor
IGF-1	Insulin-like Growth Factor 1
IGF-1R	Insulin-like Growth Factor 1 Receptor
IGFBP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
JAK	Janus Kinase
kDa	kilo Dalton
MAPK	Mitogen Activated Protein Kinase
MβCD	Methyl-β-Cyclodextrine
MUP	Major Urinary Protein
PI-3 kinase	Phosphatidyl Inositol 3'-kinase
PKC	Protein Kinase C
PL	Placental Lactogen
PLC	Phospholipase C
PPAR	Peroxisome Proliferator-Activated Receptor
PRL	Prolactin
PTP	Protein Tyrosine Phosphatase
SAM	Significance Analysis of Microarrays
SH2	Src homology domain 2
SOCS	Suppressor of Cytokine Signaling
STAT	Signal Transducer and Activator of Transcription
T3	Triiodothyronine
Ub	Ubiquitin
UPR	Unfolded Protein Response

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1 INTRODUCTION

The endocrine system is responsible for the maintenance of whole body homeostasis in mammals. Hormones are synthesized in specialized glands and then secreted into the circulation to act on target tissues. Physiological responses involving endocrine pathways, in most cases, are induced by hormone-receptor binding on target cells. Such interaction triggers a controlled cascade of intracellular events resulting in specific phenotypic changes. Many common diseases originate from specific alterations of the endocrine system, or are modulated by hormones. Endocrine disorders can either be, associated to defects in the synthesis or secretion of hormones in endocrine glands, or to defects in the responsiveness of target tissues. Diabetes clearly illustrates this statement; while type I diabetes is characterized by low insulin levels due to the destruction of beta cells in the Langerhans islets in the pancreas, type II diabetes is characterized by insulin resistance with evident hyperglycemia, despite increased circulating levels of insulin. This is not a unique phenomenon, hormone resistance appears to be common in metabolic diseases. Another example concerns leptin, a hormone synthesized in the adipose tissue that functions to regulate food intake. Obese individuals have an increased food intake, despite greatly elevated levels of circulating leptin. This may be related to insensitivity to leptin at the hypothalamic appetite regulatory center. Relatively little is known about the impact of hormone resistance on disease development. In many cases, this is due to the lack of parameters that can accurately describe the responsiveness to particular hormones on target tissues. This likely derives from our rudimentary knowledge of postreceptor signaling mechanisms and their impact on endocrine physiology. In order to understand hormonal signaling, a thorough study of both, positive and negative regulatory intracellular mechanisms is required. This will eventually lead to the emergence of a more detailed description of the endocrine system and to the development of new therapeutic strategies.

Growth Hormone (GH) constitutes a paradigm model for the study of hormonal mechanism of action. There is a wealth of information on the multiple physiological actions of GH. The main mechanisms controlling both GH secretion and its signaling pathways in target tissues have been delineated. Different disorders have been associated to either excessive or reduced GH secretion as well as altered tissue responsiveness to the hormone. Recombinant hGH is widely used to treat growth disorders in children and it is being evaluated for treating cardiovascular and metabolic disorders in adults. The pleiotropy of GH actions, its interactions with other hormonal pathways and its pharmacological potential, justify thoroughly describing the mechanisms that regulate the sensitivity to this hormone on target tissues. This thesis is focused on the study of the signal transduction mechanisms that negatively regulate GH receptor signaling pathways.

1.1 GROWTH HORMONE

GH is a polypeptide hormone mainly secreted from the anterior pituitary gland into the circulation. The first clinical observations linking the pituitary gland to a disorder were made in 1886 by Pierre Marie. He detected enlarged anterior pituitaries in acromegalic individuals. However, it was not until 1910 that studies done by Crowe *et. al*., considered the pituitary gland as an important factor for growth in the rat, frog and dog, since growth in these animals was greatly affected by hypophysectomy (1). In 1921 Evans demonstrated that the injection of saline extracts of bovine anterior pituitary gland promoted growth in normal rats (2). The purification of a protein with growth promoting effects was done in 1945 from bovine pituitaries, and called somatotropin or growth hormone (3, 4).

The human GH gene is approximately 3 Kb long and is located on chromosome 17. In humans, the GH gene is part of a gene cluster composed of 5 closely related genes that have more than 92% nucleotide sequence homology (5). The main circulating form of GH is a 22 kDa protein. Alternative splicing can give rise to a 20 kDa variant which lacks amino acid residues 32-46 (6). Two other forms, 27 kDa and 17 kDa are also present in plasma but their physiological significance is unclear (7). The GH structure consists of four antiparallel long α-helices connected in the unique "up-up-down-down" fashion. This is similar to other members of the Helix Bundle Peptide (HBP) protein family which also includes prolactin (PRL), placental lactogen (PL), interleukin- (IL) 2 to 7, IL-9, IL-11 to IL-13 and several other growth factors (8).

1.1.1 Regulation of GH expression

GH synthesis and secretion is mainly regulated by two hypothalamic peptides; the growth hormone releasing hormone (GHRH) and somatostatin. While GHRH stimulates GH transcription and secretion, somatostatin inhibits its secretion. In addition to these two factors, other hormones and physiological situations contribute to control the GH secretion by regulating the expression or activity of GHRH and somatostatin while others directly regulate GH synthesis (9).

GH, Insulin-like growth factor 1 (IGF-1), thyroid hormone (T3) and glucocorticoids can regulate GH synthesis and secretion. GH exerts a negative feedback on its own secretion by decreasing the production of GHRH and its receptor (GHRHR) and by increasing the activity of somatostatin (10, 11). High serum levels of IGF-1 (produced in response to GH) inhibit GHRH and GH release (12). Ligand-bound T3 and glucocorticoids receptors (13, 14) interact with specific response elements in the 5' flanking region of GH gene and stimulates its transcription. Serum GH levels are also increased in response to stress, sleep, exercise, hypoglycemia, amino acids, sex steroids, α-adrenergic agonists, dopamine receptors agonists and fasting, while β-adrenergic agonists, glutamate, hyperglycemia and high free fatty acids decrease GH levels (15).

In humans, GH levels are high during the first couple of weeks after birth falling gradually, and are kept low during childhood. Just before puberty, GH levels start increasing, reaching the maximum during puberty and falling gradually with age (15, 16). During puberty significant gender differences emerge in humans as well as in rats, that determine the different male/female somatic growth patterns (bone and muscle growth, fat distribution, etc) (15). Male rats exhibit secretory bursts of GH, at 3-4 hour intervals, with low or undetected levels between peaks. In females, secretion is also episodic but the peak amplitude is lower and the baseline levels are higher than those found in males (17, 18).

1.1.2 Biological effects of GH

The main physiological effect of GH is the regulation of postnatal longitudinal bone growth (19, 20). GH promotes growth through diverse and pleiotropic effects on cellular metabolism and differentiation. GH regulates carbohydrate, lipid, nitrogen and mineral metabolism (21-23) and stimulates DNA synthesis, differentiation and mitogenesis in a variety of cell types in different tissues (24-27). GH is also important in the maintenance of the immune system (28, 29), heart development and hypertrophy (30) and has been shown to act on the brain to modulate emotion, stress response and behavior (31). It is still unclear which of these actions are related to the promotion of longitudinal growth and which constitute growth-independent actions.

1.1.2.1 Somatic growth

People destined to be tall secrete GH at higher concentrations than their smaller peers, resulting in higher growth rate. A congenital failure in GH synthesis and secretion results in dwarfism, while hypersecretion induces gigantism if it occurs prepubertally and acromegaly if postpubertally (32, 33). Acromegalic patients present enlargement of hands, feet and jaw, hypertrophy of the heart and hypertension. Metabolically, these patients develop severe insulin-resistance with the symptoms of diabetes mellitus. Aging is characterized by gradual loss of GH secretion (34). Loss of GH in young adults or in children leads to significantly reduced growth rate with disturbances in some metabolic functions resulting in increased body fat and decreased muscle strength (35).

IGF-1 is an important regulator of somatic growth. IGF-1 knockout mice reach 40% of the growth observed in wild type mice (36, 37), while mice with disrupted IGF-1 receptors (IGF-1R) die at birth with a 55% reduction in weight (38). Hepatic production of IGF-1, under GH control, is the main contributor to the circulating levels (39). Liver specific deletion of the IGF-1 gene reduces circulating IGF-1 levels by 65% but does not affect growth (40). Further reduction to 15% of the normal levels can be achieved by the deletion of the Acid Labile Subunit (ALS) resulting in significant reduction of body length (41). These results suggest that a minimum circulating level of IGF-1 is required for growth and that the extrahepatic IGF-1 (also produced in response to GH) significantly contributes to body growth (42). The local administration of GH to the cartilage growth plate of hypophysectomized rats produces accelerates longitudinal

bone growth (19) with no evidence of increased circulating IGF-1. This effect is mainly due to the stimulation of chondrocyte progenitor cells in the epiphyseal growth plate (43). In summary, GH can have dual effects on target cells. Acting directly and also through locally or systemically produced IGF-1.

1.1.2.2 Regulation of Carbohydrate and Lipid metabolism

GH induces many changes in glucose and lipid metabolism. GH treatment stimulates lipolysis resulting in increased free fatty acids (FFA), glycerol and enhanced lipid oxidation. It inhibits insulin-induced suppression of hepatic gluconeogenesis and glycogen storage in liver and muscle. It also increases the circulating levels of glucose and insulin and suppresses glucose oxidation (44-46). These metabolic changes are seen in acromegaly patients resulting in a high risk of diabetes. On the other hand, GH deficiency is associated to increased fat mass and decreased lean body mass. It seems that the increase in adiposity, seen in these subjects, contributes to insulin-resistance and hyperinsulinemia (47). However, it has not yet been clarified if the resistance to insulin is related to reduced GH levels. Acute administration of GH to GH-deficient adults (GHDA) results in a reduction in insulin sensitivity with an increase in glucose and insulin concentrations in plasma while chronic administration of GH results in an increase in lean body mass, reduction of fat mass with an improvement in insulin sensitivity (44). GH also promotes nitrogen retention and regulates mineral metabolism and electrolyte balance (48).

Animal models have provided important information about the metabolic effects of GH. GH transgenic mice have an increased growth rate and IGF-1 levels, and reduced body fat. In addition to these effects, some anomalies such as impaired cardiac function, hypertension, immunity defects, and arthritic disorders are also present in these animals. Metabolically, these animals develop severe insulin-resistance with high levels of glucose and insulin (Reviewed in Bartke *et. al.*,) (49). Life expectancy appears to be inversely related to circulating GH levels. Mice overexpressing GH do not live as long as their normal siblings, showing symptoms of early aging (50). Interestingly, elevated insulin levels and poor glycaemic control, constitute risk factors for several age-related diseases in humans (51). Oxidative processes are more active in GH transgenic mice, associated with decreased activity of some anti-oxidative enzymes (52). This could negatively influence GH transgenic mice life span since it is well known that oxidative damage is a key process in cellular aging (53).

It has been established that the deletion of IGF-1 in liver (LID mice) results in high levels of GH and insulin (54), pancreatic islet hyperplasia and insulin resistance in liver, muscle and fat (41). These mice also develop diabetes in response to streptozotocin more rapidly than control mice (55). The inhibition of GH action by crossing the LID mice with GH antagonist (GHa) transgenic mice improves hepatic insulin sensitivity. This data indicate that the elevation of GH levels in LID mice have a major role in insulin resistance (56) and that IGF-1 opposes the diabetogenic actions of GH.

1.1.2.3 Gene Regulation

Transcriptional regulation is an important aspect of GH effects on target tissues. The relationship between GH-induced transcriptional regulation of IGF-1 and somatic growth has been thoroughly demonstrated. Recently, with the use of microarray technologies, our knowledge of GH-responsive genes has greatly increased. Both, long and short-term treatments with GH have been used to identify a large number of GH-regulated genes in a variety of tissues. Novel hypotheses have arisen from the analysis of these expression profiles regarding some of the GH effects that lack adequate molecular explanations (57).

The transcriptional actions of GH are exerted in a timely fashion by a network of transcription factors. How this transcriptional network orchestrates the physiological actions of GH is yet poorly understood. The discovery that the signal transducer and activator of transcription 5 (STAT5) is activated by GH (upon GHR-mediated phosphorylation) (58) represented a breakthrough in the understanding of the mechanisms behind GH transcriptional regulation. Deletion of the STAT5b gene in mice results in 27% reduction in growth rate in males and loss of sexual dimorphism in growth (59). These results further support the physiological link between STAT5 and GH-dependent effects. STAT5b is known to bind the promoter and to regulate the transcription of hepatic nuclear factor 6 (HNF-6) which in turn activates other GH regulated genes (60, 61). STAT5b can inhibit both, the transcription and the activity of peroxisome proliferator-activated receptor alpha (PPARα), possibly resulting in the induction of lipogenic actions in the liver (57, 62, 63). Other transcription factors activated by GH include the α, β, δ isoforms of the C/EBP family (64-66), HNF-1α (67), IRF-1 (68), mtTF1 (69), c-jun and c-fos (70, 71), Foxm1b (72), Runx2 (73), Hoxa1 (74) , ear-2 and TAFII28 (57). Table 1 shows genes with DNA-binding activity regulated by GH in liver and skeletal muscle, identified by microarray analysis and classified according to Gene Ontology (Flores-Morales *et. al.*, Manuscript).

Table 1. DNA-binding proteins regulated by GH**.**

Tissue	*Gene Bank*	*Name*	*Exp*
Liver	**Up regulated**		
	AB025017	zinc finger protein 36	1, 3
	AF015953	aryl hydrocarbon receptor nuclear translocator-like	1
	AY004663	nuclear factor, interleukin 3, regulated	1
	NM_012591	interferon regulatory factor 1	1
	NM_012603	v-myc avian myelocytomatosis viral oncogene homolog	1
	NM_012747	**signal transducer and activator of transcription 3**	1
	NM_012912	activating transcription factor 3	1
	NM_022671	one cut domain, family member 1	1
	NM_022858	HNF-3/forkhead homolog-1	1
	U67083	KRAB-zinc finger protein KZF-2	1
	AF092840	nibrin	2, 3
	BC029197	X-box binding protein 1	2, 3
	AK016949	SWI/SNF related, actin dependent regulator of chromatin, subfamily a, member 5	3
	NM_008416	Jun-B oncogene	3
	M22326	early growth response 1	3

Tissue	*Gene Bank*	*Name*	*Exp*
	NM_009883	**CCAAT/enhancer binding protein (C/EBP), beta**	3
	NM_010591	Jun oncogene	3
	NM_019963	signal transducer and activator of transcription 2	3
	Down regulated		
	NM_022380	**signal transducer and activator of transcription 5B**	1
	NM_023090	endothelial PAS domain protein 1	1
	U09229	cut (Drosophila)-like 1	1
	U20796	nuclear receptor subfamily 1, group D, member 2	1
	AF009328	nuclear receptor subfamily 1, group I, member 3	2, 3
	AF374266	**sterol regulatory element binding factor 1**	2
	BC036982	**thyrotroph embryonic factor**	2
	X89577	**peroxisome proliferator activated receptor alpha**	2, 3
	AK009739	Kruppel-like factor 15	3
	NM_008260	**forkhead box A3 (Foxa3) (HNF-3γ)**	3
	NM_010902	nuclear factor, erythroid derived 2, like 2	3
	NM_016974	D site albumin promoter binding protein	3
Skeletal	**Up regulated**		
Muscle	AW144714	nuclear receptor subfamily 2, group F, member 6	4
	NM_008241	forkhead box G1 (Foxg1)	4
	D45210	zinc finger protein 260	2, 3
	M22326	early growth response 1	2, 3
	NM_007564	zinc finger protein 36, C3H type-like 1	2, 3
	NM_007679	**CCAAT/enhancer binding protein (C/EBP), delta**	2, 3
	NM_008416	Jun-B oncogene	2, 3
	NM_010234	**FBJ osteosarcoma oncogene (c-fos)**	2, 3
	NM_013597	myocyte enhancer factor 2A (Mef2a)	2
	AK018370	Bcl6 interacting corepressor	3
	BC010786	cAMP responsive element binding protein 3-like 3 (Creb3l3)	3
	BC020042	core promoter element binding protein (Copeb)	3
	NM_008390	interferon regulatory factor 1	3
	NM_009029	retinoblastoma 1	3
	NM_010028	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	3
	NM_010902	nuclear factor, erythroid derived 2, like 2	3
	NM_011498	basic helix-loop-helix domain containing, class B2	3
	NM_013468	ankyrin repeat domain 1 (cardiac muscle)	3
	NM_013692	TGFB inducible early growth response 1	3
	NM_013842	X-box binding protein 1	3
	NM_020033	ankyrin repeat domain 2 (stretch responsive muscle)	3
	NM_020558	nuclear DNA binding protein	3
	Y15163	Cbp/p300-interacting transactivator, Glu/Asp-rich carboxy-terminal domain, 2	3
	Down regulated		
	BC013461	down-regulator of transcription 1	4
	NM_010866	myogenic differentiation 1	2
	BC011118	**CCAAT/enhancer binding protein (C/EBP), alpha**	2
	NM_009557	zinc finger protein 46	3
	NM_016974	D site albumin promoter binding protein	3

Experiments analyzed (Exp): 1. Hypophysectomized rats injected + GH (short term); 2. $Ghrhr^{lit/lit}$ mouse + GH 2h; 3. $SOCS2^{-/-}Ghrhr^{lit/lit}$ mouse + GH 2h; 4. Old rats + GH (injection twice per day during 1 week). In bold: transcription factors that were previously known to be GH-regulated.

1.2 GROWTH HORMONE RECEPTOR (GHR)

The existence of the GHR was first reported by Tsushima and Friesen in 1973 (75) but it was not until 1987 that the first GHR was purified, sequenced and cloned from rabbit liver (76). The rat GHR was subsequently cloned and shown to be functionally active (77). The GH receptor gene encodes a protein of 620 amino acids. The extracellular region, the hormone binding domain, contains 250 amino acids while the intracellular region contains approximately 350 residues. The GHR belongs to the Class I cytokine receptor superfamily, which also includes the receptors for erythropoietin (EPO), IL-2 to IL-9, IL-11, IL-12, trombopoietin, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (GCSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) (78).

The GHR contains five potential Asparagine (N)-linked glycosylation sites (79, 80) and twenty three potential Lysine (K)-linked ubiquitination sites. These post-translational modifications increase the molecular weight of the GHR from the expected 70 kDa to the 100-130 kDa observed in SDS-PAGE (81). It has been established that the internalization of the GHR is ubiquitin-dependent and increases upon GH binding (82). A specific domain found in the cytoplasmic region and denominated the ubiquitin-dependent endocytosis (UbE) motif (DSWVEFIELD), seems to be important for the GHR internalization (83) (Figure 1). The GHR lacks intrinsic kinase activity and the activation of cytoplasmic tyrosine kinases is crucial for their signal transduction (84).

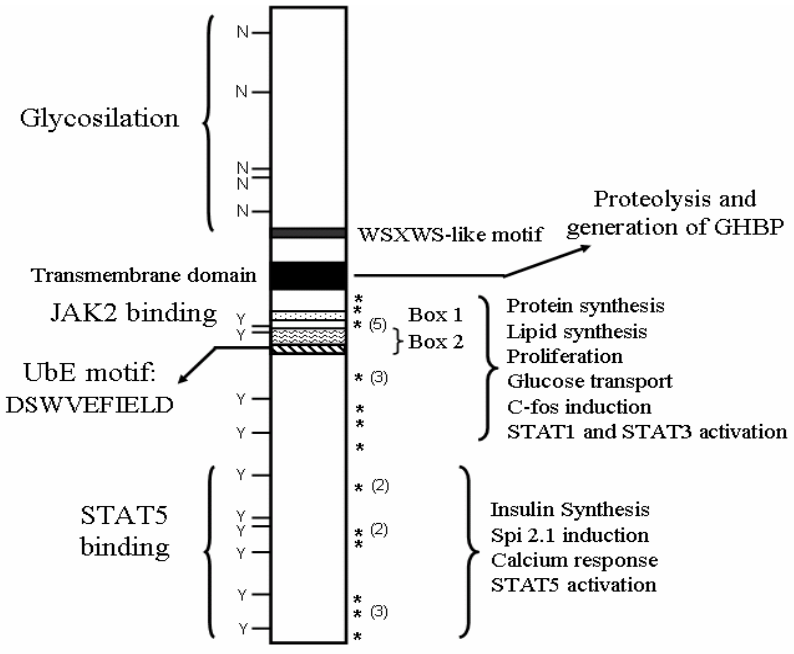


Figure 1. Structure of the Growth Hormone Receptor (GHR). The figure shows the residues subjected to post-translational modifications such as the glycosylation sites (N-linked), phosphorylation (Y residues) and ubiquitination (*) (K residues) as well as main motifs (shown as small boxes) necessary for the signaling or internalization processes. Adapted from Carter-Su *et. al.*, (85) with some modifications.

Mutations in the GHR in humans lead to the Laron Syndrome which is characterized by a short stature and other features that are found in different GH deficiency syndromes (86). Depending on the type of mutations, either the formation of the complex GHR/GH or the dimerization processes are impaired. Severe GH-resistance, characterized by very high concentrations of GH and very low levels of IGF-1 and IGFBP3, are observed in these patients. The development of GHR knockout ($GHR^{-/-}$) mice (87) has added valuable information regarding the importance of an intact GHR for GH-dependent actions. These mice are viable with an almost normal size at birth. The growth rate diminishes during postnatal life ending in around 50% reduction in adult normal body weight. Similar to Laron human patients, the circulating levels of IGF-1 and IGFBP3 are extremely low or undetectable (87, 88). In agreement with the known GH actions, these animals present a reduction of lean body mass, an increase in fat mass (49) and a reduced bone turnover and mineral density (89, 90). The $GHR^{-/-}$ mice live much longer than control mice (88).

1.2.1 GHR gene expression

The first recognized GH-responsive tissue was the liver, mainly because of the higher amount of receptors present in the hepatic cells (91). Making use of more sensitive techniques, it has been possible to quantify the amount of GHR in extrahepatic tissues such as muscle, bone, kidney, mammary gland, adipose and embryonic stem cells (92). The GHR has also been found in the human and rat brain (93) and in the immune system (94).

There are many factors that influence the expression of the GHR either at the transcriptional or translational level. These include; nutritional status, endocrine context, developmental stage (ontogeny) and various tissue/cell specific control mechanisms (95). GHR expression rises concordantly with postnatal age, being maximal at puberty. The factors controlling the developmental regulation of GH receptor expression are poorly known. Table 2 exemplifies factors that regulate GHR expression. It is important to mention that studies regarding GHR regulation, particularly by GH and insulin, can be often contradictory. This is due to differences in cell or tissue types used and/or GH doses and exposure times analyzed (acute or chronic GH effect).

1.2.2 Growth Hormone Binding Proteins (GHBP)

At least 50% of circulating GH is complexed to a high affinity binding protein (96, 97) that is a soluble form of the GHR extracellular domain. In rodents, the GHBP is generated by alternative splicing (98, 99) and in humans it is generated by proteolysis of the GHR (100). Even though, its biological significance is not fully understood, there is evidence indicating that GHBP production affects GHR levels at the membrane (101). Several studies indicate that GHBP can have both, positive effects on GH action (by preventing renal clearance of GH) and inhibitory effects (through competition for ligand with GHR, and formation of inactive heterodimers with cell surface receptors). In IM-9 cells the phorbol ester PMA induces proteolysis of GHR, leading to the

generation of GHBP and down-regulation of surface GH binding (102). Therefore, it is becoming clear that proteolysis of GHR modulates GH signaling by reducing GHR abundance at the cell surface (103).

Table 2. Regulation of GHR expression.

Factor	*Effect on GHR expression*	*System*	*Ref.*
Nutrition			
Undernutrition and fasting	↓ GHR (mRNA)	Liver, rat	(104, 105)
	↓ GHR (mRNA)	Hepatocytes, rat	(106)
Glucose starvation	↓ GHR (mRNA)	Hepatocytes, pig	(107)
Endocrine System			
GH chronic	↑ GHR (binding)	Liver, rat, pig and sheep	(108, 109)
Absence of GH (hypox)	↓ GHR (number)	Liver, Rabbit	(110)
GH acute	↑ GHR, 1h (binding) ↓ GHR, 6h (binding)	Liver, Rat	(111)
GH overexpression	↑ GHR (binding)	Liver, transgenic mice	(112)
Pregnancy	↑ GHR (mRNA and binding)	Liver, mouse	(113)
Estrogen	↑ GHR (mRNA)	Liver, rat	(114)
Dexamethasone	↓ GHR (mRNA)	Liver, rat	(114)
Insulin	↑ GHR (mRNA and protein) ↓ GHR surface	HuH7 cells	(115)
	↓ GHR (binding)	H4 cells	(116)

The arrow indicates the effect on GHR expression: ↑: increase; ↓: decrease. Hypox: hypophysectomy

1.3 GH-DEPENDENT SIGNAL TRANSDUCTION PATHWAYS

1.3.1 GHR dimerization

The binding of GH to the extracellular domain of GHR on target cells, promotes homodimerization of the receptor. It is believed that this process results in conformational changes on the receptor that initiate GH-dependent intracellular signaling pathways. Crystallographic analysis demonstrated that one GH molecule, through two GHR-binding sites (a high affinity site or site 1, and a lower affinity site or site 2), binds two GHR molecules (117-119). The interaction between the GH and its receptor is sequential. First, GH binds to one GHR molecule through site 1 and then to a second receptor through site 2. The second binding is only possible if the first GHR is already bound to the GH molecule (119). Some recent studies suggest that the GHR can dimerize in the endoplasmic reticulum and at the cell surface in the absence of ligand (120, 121).

Several studies have demonstrated the importance of GHR dimerization in GH-dependent actions. GH analogs that cannot induce dimerization of GHR are inactive (122), the disruption of site 2 on the GH molecule generates GH antagonistic activity *in vivo* (123) and *in vitro* (119) and use of monoclonal antibodies that prevent GHR dimerization antagonizes GH-dependent cellular proliferation (117). GHR lacking the intracellular part inhibits the function of the full-length receptor due to the formation of nonproductive dimers (124). However, monoclonal antibodies directed to the extracellular part of the receptor and that are able to induce dimerization fail to induce signal transduction (125), suggesting that the dimerization by itself is not enough to initiate signaling and that conformational changes induced by GH are also important.

1.3.2 Activation of cytoplasmic kinases in response to GH

GHR lacks intrinsic enzymatic activity and relays the recruitment/activation of cytoplasmic tyrosine kinases for the signal transduction process (126). The most important tyrosine kinase mediating GH actions is JAK2 (127, 128). JAK2 activation subsequently results in activation of several non-receptor tyrosine kinases, which can initiate different, but in many cases interconnected, signaling pathways. JAK2 associates to GHR through the so-called Box1 which is located proximally to the transmembrane region. The crucial importance of JAK2 has been demonstrated by mutagenesis of GHR's Box1 and deletion of JAK2. In both cases, GHR is rendered inactive (129, 130).

It was believed that GH binding to and dimerization of GHR recruited JAK2 proteins to the receptor. However, there is evidence indicating that JAK2 is constitutively associated with the receptors, and it is very likely that ligand binding stabilizes the preformed receptor-JAK complex (131). GH induces conformational changes in GHR that result in transphosphorylation and catalytic activation of JAK2's kinase domain (132). Maximal JAK2 phosphorylation requires the presence of at least one-third of the GHR's cytoplasmic tail (129). Once JAK2 is activated, it phosphorylates GHR at multiple tyrosine residues (133). Phosphorylated GHR-JAK2 complexes provide multiple docking sites for SH2-containing signaling molecules or phosphotyrosine binding (PTB) motifs.

JAK2 belongs to the family of **J**anus **A**ssociated **K**inases which also include; JAK1, JAK3 and Tyk2 (134). GH is also able to induce the phosphorylation of JAK1 and JAK3 (135, 136), but with much lower activation levels than for JAK2 (131). Although, there is no evidence regarding Tyk2 activation by GH, association of Tyk2 to the receptor has been detected in human liver cells (137) suggesting that GH may also utilize this kinase.

GH recruits, through the activation of JAK2, other non-receptor tyrosine kinases such as c-Src, c-Fyn (138) and FAK (139). The first two proteins belong to the Src family PTKs which is mainly involved in signaling pathways that regulate cell cycle, cell adhesion, migration, cell proliferation and differentiation (140). Although the exact role for the activation of these proteins by GH is unclear, some studies suggest that other

GH-dependent proteins such as FAK, p130Cas and c-Cbl can act as substrates for c-Src. FAK is activated in response to GH in CHO cells stably transfected with the rat GHR resulting in the phosphorylation of two FAK-associate substrates, paxillin and tensin (139). The activation of FAK induces reorganization of the actin cytoskeleton (141, 142).

1.3.3 Signaling Pathways

Upon activation, the GHR/JAK2 complex triggers the phosphorylation of various cytoplasmic proteins. Many of these pathways are shared by other cytokines and growth factors and have been characterized *in vitro*. But, their significance in the hormone's *in vivo* actions is largely unknown. One of the GH activated pathways with clear physiological significance is the activation of STAT5. STAT5 is associated to GHR and becomes rapidly phosphorylated (143) in response to GH.

1.3.3.1 JAK-STAT Pathway

Shortly after phosphorylation of the GHR/JAK2 complex, STAT5a/b proteins are recruited to the activated complex. JAK2 then phosphorylates STAT5a/b tyrosine residues. It has also been shown that STAT5a can be serine phosphorylated via mitogen activated protein kinase (MAPK) pathway (144). Once STAT5 proteins are phosphorylated, they dissociate from the receptor, dimerize (homo- or hetero-dimerization) through the SH2 domains in each STAT molecule and migrate to the nucleus where they bind specific DNA response elements (58) and consequently regulate gene transcription (Figure 2).

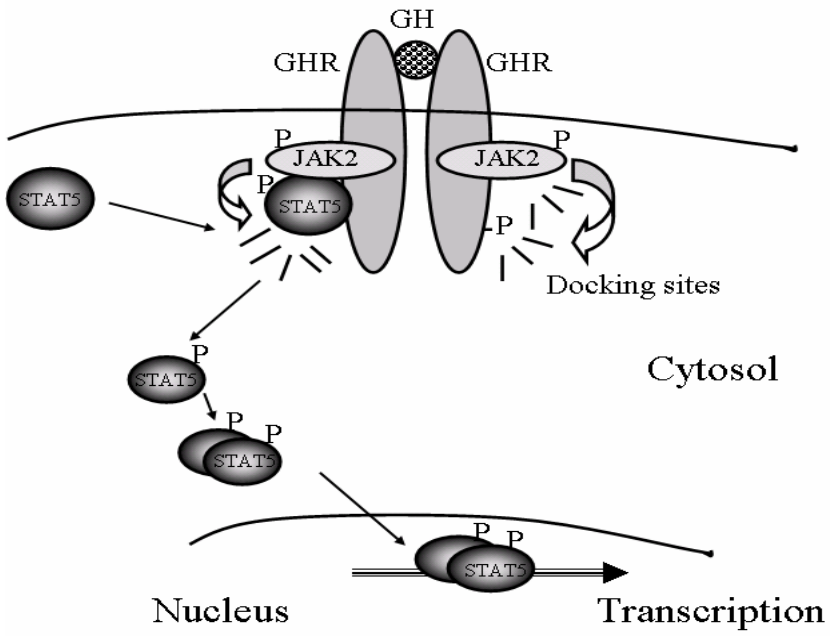


Figure 2. GH-dependent JAK2/STAT5 signaling pathway. GH binding to GHR on target cells induces tyrosine phosphorylation of GHR/JAK2, which is followed by recruitment of STAT5 proteins to the vicinity of the GHR. Phosphorylation of STAT5 allows the dimerization and translocation of STAT5 to the nucleus where they regulate gene expression.

STAT5 belongs to the family of **S**ignal **T**ransducers and **A**ctivators of **T**ranscription (STAT) proteins which are latent cytoplasmic transcription factors. STAT proteins are the only known family of transcription factors whose activity is regulated by tyrosine phosphorylation (145). Up to now, seven mammalian STATs have been identified; STAT1 to STAT5a, STAT5b and STAT6 (145, 146). The two STAT5 isoforms are encoded by two different genes, which have ~95% homology in their coding sequence. They posses both overlapping and distinct functions in GH signal transduction (147). Besides STAT5a and STAT5b (148-152), GH is also able to activate STAT1 and STAT3 (153-155).

STAT proteins contain five domains (Figure 3), the N-terminal domain, the DNA binding domain, a coiled-coil domain, a SH2 domain and the transcriptional activation domain (located at the C-terminus of the protein). The presence of a tyrosine at the C-terminus, which is the substrate for JAK kinases, is also conserved. STAT proteins bind the cytokine receptors through the SH2 domain. All the structural domains on STAT proteins are important for their transcriptional activity. The SH2 domain and the N-terminus (residues 1-120) are crucial for the stability of the STAT-DNA interaction. The coiled-coil domain forms a hydrophilic interface for potential protein interactions. Removing the 50 C-terminal residues allows dimerization and DNA binding but does not result in transcriptional activation (156-158).

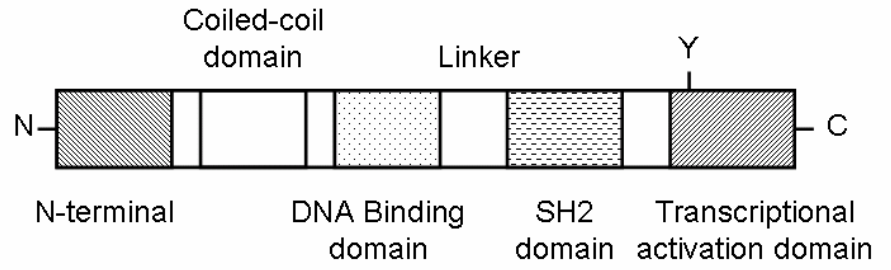


Figure 3. Structure of STAT proteins. The binding of STAT proteins to GHR is mediated by the SH2 domain. The tyrosine in the transcriptional activation domain becomes phosphorylated by JAK2 and allows the dimerization of STAT proteins. The SH2 domain, the N-terminal domain and the coil-coiled domain contribute to DNA-binding. The C-terminal domain has transactivation properties.

Gene-targeting in mice has made possible the elucidation of the molecular role of each member of the STAT protein family (Summarized in Table 5).

Table 5. Phenotypical characteristics of mice lacking specific STAT proteins.

STAT	***Phenotype of the null mice***	***Ref***
STAT1	Impaired responses to interferons; impaired growth control and increased susceptibility to tumors	(159, 160)
STAT2	Impaired responses to interferons	(161)
STAT3	Embryonic lethality; impaired response to pathogens and multiple defects in adult tissues including cell survival	(162)
STAT4	Impaired TH1 cell differentiation caused by loss of IL-12 responsiveness	(163, 164)
STAT5a	Impaired mammary gland development owing to loss of PRL responsiveness	(165)
STAT5b	**Impaired growth due to altered Growth Hormone responsiveness**	(59)
STAT6	Impaired TH2 cell differentiation caused by loss of IL-4 responsiveness	(166-168)

The analysis of knockout mice shows that STAT5b is directly involved in longitudinal growth and in the sexually dimorphic responses to GH in hepatic gene expression. STAT5b deficiency ($STAT5b^{-/-}$) results in 27% reduction in body growth in males (59), elevated GH plasma levels, reduced circulating IGF-1 and obesity. There is also a loss of gender dependent responses associated with the dimorphic pattern of GH secretion. The expression of hepatic male specific genes such as major urinary protein (MUP), were decreased to the same levels as female wild type mice while the expression levels of female predominant genes were higher than those found in wild type females. Moreover, STAT5b is required for the GH-dependent hepatic expression of IGF-1, IGFBP3, ALS, SOCS1, SOCS2, SOCS3 and CIS (169-171) as well as the stimulation of lipolysis in adipose tissue (172).

The phenotype of $STAT5a^{-/-}$ resembles the one found in PRLR-deficient mice (173). Some characteristics of these animals include the impaired mammary gland development and lactogenesis without any defect in body growth, suggesting that STAT5a is necessary for PRL function but not GH action. The cooperation of STAT5a and STAT5b has been demonstrated in GH-dependent expression of ALS (174) and Spi 2.1 (152, 175, 176). The double knockout ($STAT5a/b^{-/-}$) reveals additional phenotypes that are not observed in the single knockouts, namely female infertility and a more pronounced defect in growth (177).

Despite the high homology of the two STAT5 isoforms, they have clearly distinct roles (177). This suggests that their specificity may be influenced by factors such as relative abundance of these proteins in different tissues and probably selective interactions with other proteins and transcription factors. It was established that the expression of STAT5b is 10-fold greater than that of STAT5a in liver and also significantly higher in males compared to females (148, 178). Even though, there is evidence that other STAT-interacting molecules may facilitate STAT recruitment to receptor complexes

and therefore enhance signaling (179, 180), these mechanisms are not yet described in relation to STAT5a or STAT5b.

The activation of STAT proteins by GH may also be cell type specific. For example, GH is unable to stimulate STAT1 and STAT3 activity in IM-9 lymphocytes despite the ability of IFN-γ to activate these STAT proteins in the same cell line (181, 182). In 3T3-F442A fibroblast GH regulates the expression of *c-fos* through the activation of STAT1 and STAT3 (155, 183). This specificity may be due to the interaction of STATs with other tissue specific transcription factors in the regulation of complex response elements of a particular gene.

1.3.3.2 Other signaling pathways

It has been demonstrated that GH activates several signaling pathways other that STAT5. The exact role and the relevance of each of these pathways in GH physiological effects are unclear, since many of these pathways are also activated by several growth factors and cytokines (Figure 4). A comprehensive review has been published regarding GH-activated signaling pathways (184). Below, there is an overview of some GH-activated pathways. In most of the cases described, the data has been obtained from *in vitro* systems.

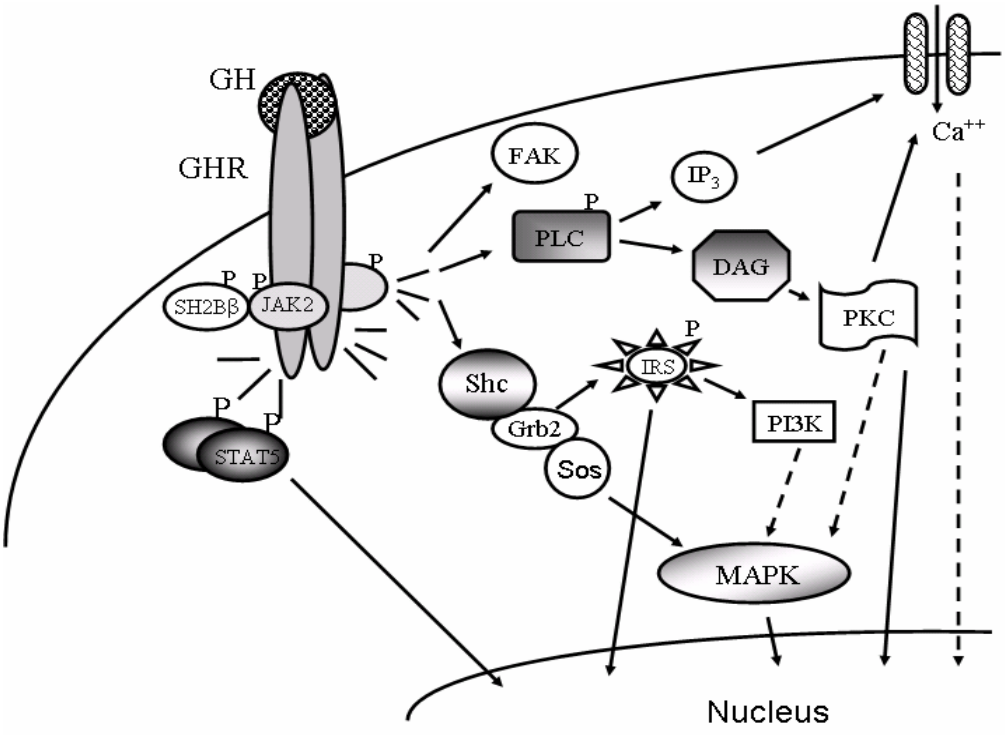


Figure 4. GH-dependent signaling pathways. Besides JAK2/STAT5 pathway, GH is able to signal through the MAPK kinase pathway, PLC and IRS, among others. All these signal pathways have as a final result the regulation in gene transcription. Adapted from Carter-Su *et. al.*, (185) with some modifications.

1.3.3.2.1 MAPK kinase pathway

MAPK kinases have an important role in the regulation of gene transcription, cellular proliferation and prevention of apoptosis. The MAP kinase pathway involves activation of protein-serine/threonine kinases that are highly conserved in evolution.

GH has been shown to activate p44/42 MAPK through the sequential activation of Shc, growth factor receptor bound 2 (Grb2), son of sevenless (SOS), Ras, Raf, MAP/ERK kinase (MEK) and MAPK (186, 187). The binding of Shc to the phosphorylated residues in JAK2 and GHR is the first step in GH-dependent MAPK kinase signaling pathway. Once Shc is phosphorylated it binds and activates Grb2 which then activates SOS. SOS activates Ras, Raf, MEK and MAPK (188). GH can also activate p70S6K, p90RSK, Sap-1a, phospholipase A2 (PLA2), c-jun, p62TCF/Elk1) and STAT molecules through the activation of p44/42 (132, 189, 190).

1.3.3.2.2 Phospholipase C (PLC)/PKC/Ca^{2+} pathway

The hydrolysis of phosphatidylinositol 4,5 bisphosphate catalyzed by PLC results in the production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) which act as second messenger molecules. IP3 increases intracellular Ca^{2+} levels while DAG activates PKC.

The PLCγ has been shown to be phosphorylated in response to GH through its direct binding to GHR/JAK2 complex (191). The activation of pathways downstream PLC seems to be also cell type specific. For example, GH induces the production of DAG and IP3 in proximal tubule cells (192) while it induces only the production of DAG in β-islet cells (193). PKC depletion reduces some GH-dependent effects such as expression of c-fos (71, 194, 195), the increase in intracellular calcium (196) and activation of p44/42 MAPK (197).

Two mechanisms are used by cells to increase intracellular calcium concentration; calcium release from intracellular stores and calcium influx from the extracellular space through voltage-dependent calcium channels. It has been shown that GH uses both mechanisms (198) to increase the concentration of intracellular calcium in several cell lines (196, 199-201). Using blockers of L-type calcium channels (verapamile and nimodipine) it was shown that GH also activates the L-type calcium channel (202).

1.3.3.2.3 Insulin receptors substrates (IRS) and PI-3 kinase

IRS represents another pathway by which GH elicits some of its effects. GH and Insulin have some common cellular effects such as stimulation of glucose transport, protein synthesis, amino acid transport, lipogenesis, gene regulation, differentiation, mitogenesis, prevention of apoptosis and reorganization of the cytoskeleton network (21, 203, 204). It is expected that GH and Insulin share some signaling pathways. IRS-1, -2 and -3 proteins associate with JAK2 via an adaptor molecule (Grb2 or CrkII) (186, 189, 205) and become phosphorylated by GH (206). Phosphorylation of IRS creates docking sites for other SH2-containing signaling molecules such as PI-3 kinase and

SHP2 (184). Some of these associated proteins are also activated or phosphorylated by GH. PI-3 kinase phosphorylates inositol lipids generating polyphosphoinositides PtdIns-3-P, PtdIns-3,4-P2 and PtdIns-3,4,5-P3 which are involved in signal transduction and vesicular trafficking (184). The role of GH has been shown in processes such as cytoskeletal reorganization, cellular metabolism, cell proliferation and survival mediated by PI-3 kinase (207, 208).

1.4 NEGATIVE REGULATION OF GH SIGNALING

The activation of GH-signaling pathways is rapid and transient. Maximal induction of JAK2 tyrosine phosphorylation is achieved a few minutes after GH stimulus (Figure 5). *In vitro*, the activation phase is followed by a period of desensitization when maximal activation of GH signaling cannot be achieved.

There are several levels at which the down regulation of GHR-mediated signaling pathway can be achieved: receptor turnover (internalization/ recycling/degradation), inactivation of positive regulators by dephosphorylation, degradation of signaling intermediates, action of negative regulators and very likely other mechanisms not yet described.

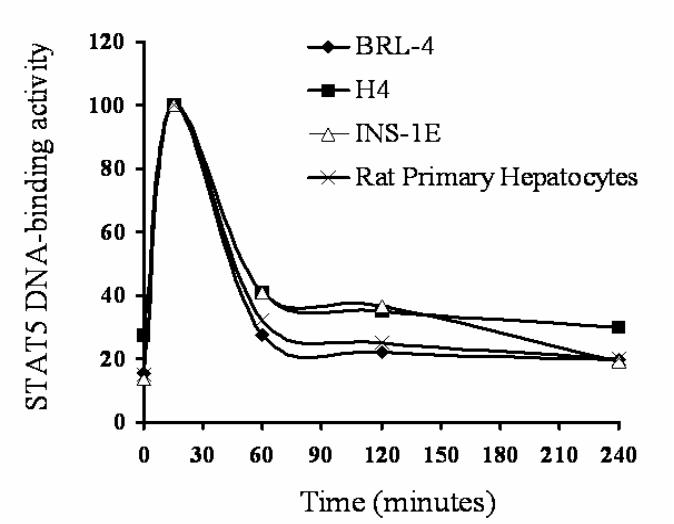


Figure 5. GH-dependent activation of STAT5 DNA-binding activity in different cellular models.

1.4.1 GHR down regulation

The regulation of the number of active GHRs on cell membranes is a key mechanism to control GH sensitivity. It is known that the removal of cell surface receptors is an early step in the termination of GH-dependent signaling. This is mediated through internalization/endocytosis. The concentration of GHR is regulated by multiple factors (see table 2), including GH itself. This regulation is achieved at multiple levels from transcription to protein synthesis and transport to the membrane. Almost all the newly synthesized GHR is converted to the mature form and directed to the cell membrane (209) but a minor portion can be also directed to the lysosomal compartment. At the

plasma membrane, the receptors can be endocytosed, transport to the endosomes, where they can be recycled to the membrane or targeted for degradation in the lysosome in an ubiquitin-proteasome-dependent manner. A portion of GHR at the plasma membrane can be cleaved resulting in the formation of the high affinity GHBP. The potential role for the GHBP has already been addressed in this thesis.

1.4.1.1 The Ubiquitin-Proteasome Pathway

Degradation of proteins by the ubiquitin-proteasome pathway requires two steps; 1) the covalent attachment of ubiquitin (Ub) to the target protein (ubiquitination) and 2) the degradation of the ubiquitinated protein by the 26S proteasome complex with the release of ubiquitin, which can then be reused.

Ubiquitination is a post-translational modification that results in the covalent attachment of Ubiquitin to lysine residues on target proteins (Figure 6). This process is the result of the sequential action of three classes of enzymes; **E1** or ubiquitin-activating enzyme, **E2** or ubiquitin conjugating enzyme and **E3** or ubiquitin ligase protein (210), which in most of the cases is a protein complex. Ubiquitination starts with activation of Ub by the action of E1 through the formation of a bond between the carboxy-terminal glycine of the Ub and a reactive cysteine present in E1. Then one of the E2 enzymes transfers the activated Ub moiety from E1 to E2. The attachment of Ub to the target protein is mediated by a specific factor (E3 proteins) which interacts with the target protein and E2. The covalent attachment of Ub to the target protein is made via an amide linkage to the ε-amino group on an internal lysine residue in the target protein.

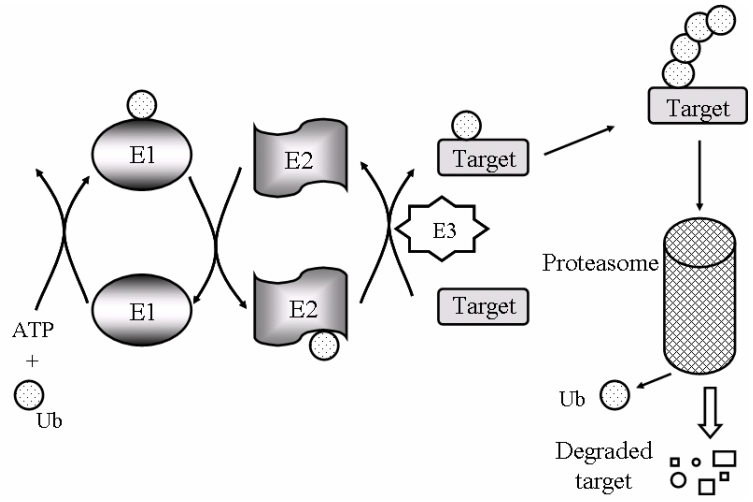


Figure 6. Ubiquitination and degradation of proteins through the ubiquitin-proteasome system.

Substrate specificity in the ubiquitination process is largely determined by the E3. This statement is supported by the number of E1, E2 and E3 enzymes present in the mammalian genomes. So far, only two E1 isoforms have been found, referred to as E1a and E1b (211), in contrast to over 30 E2 proteins (212) and approximately 500 predicted E3 ubiquitin ligases (213). Ubiquitin ligases (E3) can be divided into 4 subclasses according to some common domains; the HECT domain, RING finger, PHD

finger and the U-box (210, 214). It is now known that the recognition of target proteins by the E3 enzymes involves the action of more than one protein, usually organized into a protein complex (210, 214).

The 26S proteasome is a multicatalytic enzyme complex found in the nucleus and in the cytoplasm of all eukaryotic cells that is in charge of degrading ubiquitinated proteins (215). The proteasome consists of a 20S core particle associated with one or two 19S regulatory particles. The 19S subunit is able to bind the polyubiquitin chain and cleaves it from the target protein. The target protein is then denatured (unfolded) and fed into the proteolytic core (216), where it is degraded in a progressive manner resulting in peptides of 3-25 amino acids in length (217).

1.4.1.2 Internalization of the Growth Hormone Receptor

The first evidence of GHR ubiquitination was provided by Cheung *et. al.* in 1987 (76). By that time, it was already established that the GHR is constitutively internalized in adipocytes (218, 219). Internalization is required for GH-induced signaling down regulation (220) and is dependent on phenylalanine 346 residue in the GHR. GH binding increases GHR ubiquitination and its internalization rate. Both of these effects require an intact ubiquitin system (82, 221) and cannot proceed in cells that lack the ubiquitin activating enzyme, E1. Ubiquitin-dependent GHR endocytosis relies on GHR UbE motif, which contains the phenylalanine 346. Despite these findings, it seems that the internalization process can proceed in the absence of GHR ubiquitination. This comes from mutational studies showing that GHR endocytosis was not affected by replacement of lysine residues (to arginine) in the GHR cytoplasmic region (209). Interestingly, an intact ubiquitination system is still necessary for internalizing such GHR variants. A truncated GHR form containing residues 1-349 can be internalized independently of ubiquitination. In this case a different mechanism acts that uses a di-leucin motif in the receptor (222). The significance of this mechanism is unclear since these forms of the receptor are signaling deficient (86).

Inhibition of the proteasome also results in inhibition of GH-induced GHR internalization and degradation, but only of the GHR full length form. Short forms, lacking residues 370-620 of the intracellular domain are internalized and recycled to the membrane despite proteasomal inhibition. In the case of the full length GHR, it has been demonstrated that that the cytoplasmic part of the receptor is degraded shortly after internalization (223). GHR is internalized by both Clathrin-coated pits (224) and caveolae (225). GHR ubiquitination takes place at the plasma membrane before endocytosis which coincides with the recruitment of GHR into the clathrin-coated pits (209). It is not known yet how the UbE motif mediates GHR ubiquitination. Interaction with proteins acting as ubiquitin ligases, through this motif, could be one of the mechanisms enabling ubiquitination of GHR or its associated proteins. The formation of these protein complexes may facilitate the association of GHR with clathrin-coated pits and the endocytic machinery.

A very strong indication that the ubiquitin-proteasome system is involved in GHR negative regulation comes from the fact that proteasome inhibition prolongs GHR phosphorylation and JAK2/STAT5 activation (226-231). Internalization itself is not sufficient to downregulate the GHR since GHR/JAK2 complex is still active in the endosomes (226). From the evidence presented above, one can suggest a model for GHR internalization and degradation pathway (Figure 7). However, this model has to be taken with caution since most of the studies were performed using only one cell system (transfected Chinese Hamster Ovary cell variants) and the relevance *in vivo* has not been addressed yet. It is important to note that GHR associated proteins might also play an important role in the internalization of the receptor.

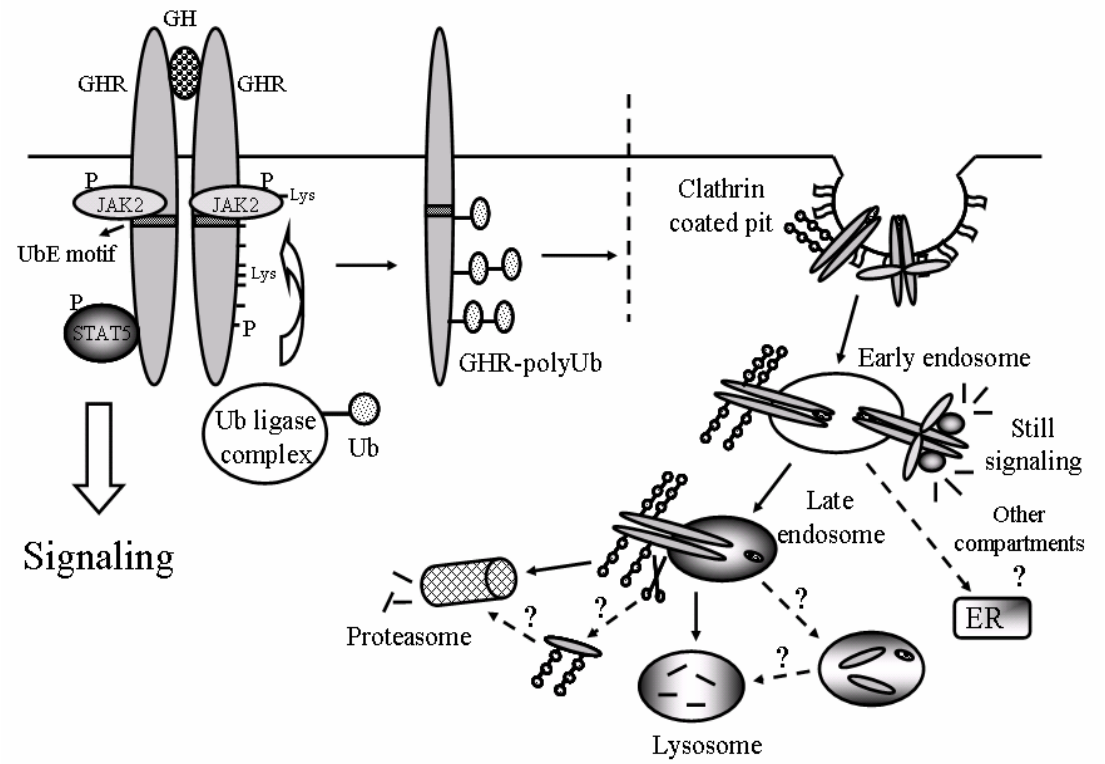


Figure 7. GHR internalization. Upon GH binding, the GHR is ubiquitinated in lysine residues present in its intracellular domain. The UbE motif is important for this process. Ubiquitination of GHR and possible other associated proteins precedes internalization which is mainly mediated by clathrin coated pits (vesicles). Final inactivation of GHR is achieved by GHR degradation in the lysosomes and/or the proteasome.

1.4.2 Actions of Negative Regulators

1.4.2.1 The SOCS Family of Proteins

The Suppressors of Cytokine Signaling (SOCS) family of proteins was identified in 1997 by three different research groups (232-234). They act as negative regulators of the main cytokine-activated signaling pathway, the JAK/STAT cascade. SOCS family comprises at least eight proteins; cytokine-inducible SH2 protein (CIS) and SOCS-1 to SOCS-7, which share similar domain architecture. They have a variable amino-

terminal region; a central SH2 (Src-homology 2) domain and a conserved carboxy-terminal motif denominated the SOCS box (Figure 8). It has been suggested that pairs of these proteins are more closely related to each other than to other members of the family; SOCS1 and SOCS3 have a high homology in their sequence, SOCS2 shares approximately 35% amino acid identity with CIS (232, 234); SOCS4 and SOCS5 have also certain degree of homology and finally SOCS6 shares homology with SOCS7 (235). In addition to the SH2 domain and the SOCS box, SOCS1 and SOCS3 contain a kinase inhibitory region (KIR), at the N-terminal part, that has not been described for other family members (236, 237). The SOCS box has also been identified in other proteins that lack SH2 domain but instead have other domains that can mediate protein-protein interactions (ankyrin repeats, WD40 repeats or SPRY domains).

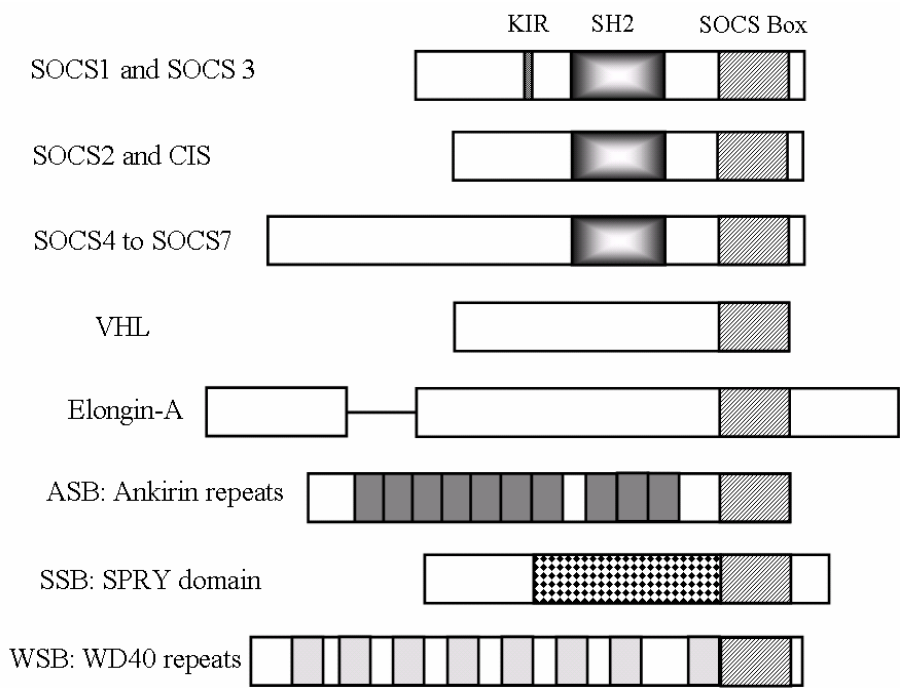


Figure 8. Structure of SOCS proteins. The Kinase Inhibitory Region (KIR) only found in SOCS1 and SOCS3 is shown as a small box at the N-terminal region. Adapted from Elliot and Johnston (238) with some modifications.

In general, SOCS proteins levels are constitutively low but their expression is rapidly induced by stimulation with different cytokines or growth factors. SOCS mRNA expression seems to be dependent on the activity of STAT proteins indicating that SOCS proteins modify cytokine action through a negative feed back loop. Table 6 summarizes the regulation of SOCS expression by several cytokines and factors, including GH.

Table 6. Factors that induce SOCS expression.

SOCS	***Induced by***
CIS	**GH**, PRL, Leptin, EPO, TSLP, IL-2, IL-3, IL-6; IL-9, IFNα, TNFα, LPS
SOCS1	**GH**, PRL, Insulin, CNTF, cadiotropin, TSH, EPO, TPO, TSLP, G-CSF, GM-CSF, IL-2. IL-4, IL-6, IL-7, IL-9, IL-13, IFNα/β, IFNγ, LIF, TNFα, thyrotropin, CXCL12.
SOCS2	**GH**, PRL, Insulin, EPO, CNTF, cadiotropin, IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10 IFNα, IFNγ, LIF, Estrogen
SOCS3	**GH**, PRL, Insulin, Leptin, CNTF, EPO, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-6,IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IFNα, IFNγ, LIF, LPS, TPO, CXCL12.
SOCS4	EGF
SOCS5	IL-6, EGF
SOCS6	Insulin
SOCS7	Unknown

For references, see Fujimoto *et. al.*, (239), Alexander WS (240) and Greenhalgh *et. al.*, (241).

It has been shown that SOCS proteins mainly target the JAK/STAT pathway thereby regulating the action of multiple cytokines. In recent years, several research groups have studied the role of each SOCS protein using *in vitro* systems. It is clear that SOCS proteins, when overexpressed in diverse cell lines, inhibit the action of a wide range of cytokines. SOCS effects can be demonstrated by the reduction of JAK and STAT phosphorylation, STAT dimerization, nuclear translocation and STAT transcriptional activity (232, 233, 242). Table 7 summarizes cytokine and growth factors whose signaling pathways can be inhibited by SOCS proteins.

Table 7. Cytokine and growth factors signaling inhibited by SOCS proteins

SOCS	***Demonstrated inhibition in the signaling activated by***
CIS	**GH**, PRL, EPO, IL-2, IL-3
SOCS1	**GH**, PRL, Insulin, Leptin, EPO, TPO, TSLP, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-15, IFNα/β, IFNγ, LIF, TNFα, IGF1
SOCS2	**GH**, PRL, IGF-1, IL-6, LIF
SOCS3	**GH**, PRL, Insulin, Leptin, EPO, IL-2, IL-3 IL-4, IL-6, IL-9, IL-10, IL-11, IFNα/β, IFNγ, LIF, IGF-1.
SOCS4	EGF
SOCS5	IL-4, IL-6, LIF, EGF
SOCS6	Unknown
SOCS7	PRL, GH, Leptin, Binding to Nck

For references, see Fujimoto *et. al.*, (239), Alexander WS (240) and Greenhalgh *et. al.*, (241).

The redundancy and promiscuity of SOCS proteins are clearly observed in Tables 6 and 7. It is also evident that in some cases a closed feedback loop is not achieved. For example, CIS is induced by a wide range of cytokines, however, CIS inhibitory effects are restricted to fewer cytokines. SOCS proteins inhibit the effects of cytokines other than the ones inducing their expression (e.g. SOCS2 and IGF-1 or SOCS5 and IL-4). Evidence also indicates that some receptors that do not belong to the cytokine receptor

family (e.g. insulin receptor, chemokine receptor) induce SOCS expression (243-245). In summary, SOCS proteins provide a way for multiple factors to regulate the activity of specific cytokines.

The cross-talk between pathways and the redundancy in SOCS action complicate the understanding of the biological significance of their inhibitory actions. However, the analysis of genetically manipulated mice has revealed essential pathophysiological roles for SOCS proteins (Table 8).

Table 8. Phenotypes associated with genetic modification of SOCS.

SOCS	***Phenotype***	***Mechanism altered***	***Ref.***
CIS	Tg: Growth retardation (10%), T cell function altered, impaired mammary gland development.	**GH**, PRL, IL2, IL-15	(246)
	Ko: No obvious phenotypical changes.		(247)
SOCS1	Tg: Altered T cell homeostasis and thymocyte development.	PRL, IFNγ, TNFα, insulin	(248)
	Ko: growth retardation, perinatal death, liver necrosis, organ infiltration, lymphopenia. Enhanced insulin sensitivity		(249-252)
SOCS2	Tg: Mild excessive growth.	**GH, IGF-1**	(253)
	Ko: Gigantism, elevated extrahepatic IGF-1, lower MUP.		(254)
SOCS3	Tg: Embryonic lethality, anemia	LIF, IL-6, EPO	(255-257)
	Ko: Embryonic lethality, placental insufficiency.		
	Liver specific Ko: IL-6 and INF-γ hyperresponsives		
SOCS5	Tg: Altered Th1 and Th2 cell balance	IL-4	(258)
SOCS6	Ko: Mild growth retardation	**IGF-1**/insulin	(259)
SOCS7	Ko: Mild growth retardation (7-10% smaller). Hydrocephaly		(260)

1.4.2.1.1 Mode of Action

Different mechanisms can explain the mode of action of SOCS proteins. They can act as (i) kinase inhibitors of JAK proteins, (ii) binding competitors against positive regulators and (iii) part of ubiquitin ligase complexes to promote the degradation process.

SOCS1, through its SH2 domain, binds the Y1007 within JAK2´s activation loop resulting in reduced JAK2 and STAT5 phosphorylation (234, 261, 262). It has been shown that KIR is required for SOCS1 action on JAK2 (261). Accordingly, the N-terminal region of SOCS1 is interchangeable with that of SOCS3, but not with that of SOCS2 or CIS (262). It has been proposed that, once SOCS1 binds to phosphorylated JAK2, it blocks the JAK2 kinase activity through the KIR (261) preventing the access of other JAK2 substrates. SOCS3 mainly binds the activated cytokine receptor thereby blocking the access of STAT proteins, but it can also bind JAK proteins (263). Similarly to SOCS1, SOCS3 inhibits the kinase activity of JAK2 through its KIR (236).

CIS associates to STAT-binding sites on the activated cytokine receptor through its SH2 domain (264-266). Since no further activity has been attributed to CIS, it is believed that it inhibits signaling by competitively masking the STAT-binding sites of the activated receptor. The mechanism of action of SOCS2 is not yet known. However, it has been suggested that SOCS2 uses a similar mechanism to the one used for CIS (Binding to the activated cytokine receptor and blocking STAT5 binding). In support of this hypothesis, it has been shown that SOCS2 interacts with the intracellular domain of IGF-1 receptor (267), GHR (through the Y595) (253) and PRLR (268). In the latter case, SOCS2 suppresses SOCS1 inhibitory effects in response to PRL suggesting that SOCS2 may act to restore cellular sensitivity to cytokines by suppressing the effects of other SOCS (269). SOCS2 can also have negative effects on other cytokines, especially GH (265).

Another model for SOCS mechanism of action has been described based on the SOCS box domain. The role of the SOCS box in the inhibitory actions of SOCS proteins has been overlooked in overexpression systems, where the absence of the SOCS box does not affect SOCS1 and SOCS3 binding ability or their inhibitory action (237, 262). However, bioinformatic analysis, showed that a large number of proteins contain the denominated SOCS box motif (Figure 7) (235), suggesting that it has an important functional role. Furthermore, mice lacking the SOCS box motif of SOCS1 have a phenotype similar to $SOCS1^{-/-}$ mice, indicating that the SOCS box is indeed essential for complete activity of SOCS1 (270).

Supporting a key role of the SOCS box, is the finding that SOCS1 and SOCS3 associate with a complex containing Elongins B and C (Elongin BC) through their respective SOCS box (271, 272). Elongin BC associates with the α domain (which contains a SOCS box) of von Hippel-Lindau (VHL) tumor-suppressor gen product, the Cullin family member Cul2 and the ring finger Rbx1 (Roc1) to assemble an E3 ubiquitin-ligase complex (273). VHL ubiquitin ligase activity targets HIF-1 for proteasomal degradation. Given that SOCS proteins contain SH2 domains, they could act as adapters that bring ubiquitin ligases into the vicinity of phosphorylated signaling proteins and cause their ubiquitination and degradation by the proteasome. Indeed, it has been shown that SOCS1, through its SOCS box, induces the proteasomal degradation of guanine nucleotide exchange factor Vav (274), oncogen Tel-JAK2 (275, 276), JAK2 (Figure 9) (277) and IRS1/2 (278). Ubiquitin-dependent degradation of IRS1/2 can also be mediated by SOCS3 (278).

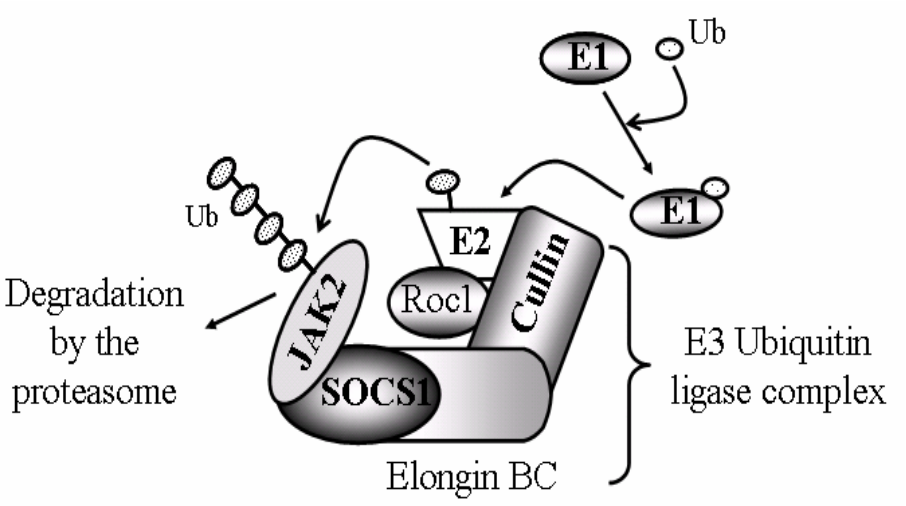


Figure 9. Ubiquitin ligase action of SOCS proteins. JAK2 degradation has been demonstrated to be mediated by SOCS1 through the SOCS box of SOCS1 and the formation of an ubiquitin ligase complex containing Cullin and Roc1. Adapted from Wormald and Hilton (279) with some modifications.

SOCS proteins can themselves be targeted for ubiquitination and proteasomal degradation. Contradictory results exist regarding the mechanism controlling the stability of CIS, SOCS1 and SOCS3. It seems that Elongin BC complex can have dual actions. It can promote SOCS proteins degradation as has been shown for CIS (280, 281), SOCS1 (271, 282) and SOCS3 (283), while in other cellular systems, it can also stabilize SOCS3 (272, 284) and SOCS1 (237, 271, 272).

A general model for SOCS proteins action cannot be suggested at this time. It seems that the mechanism whereby any particular member of the family exerts its inhibitory effect is cell type specific and cytokine-dependent. The cause of this may lie in the action of still unidentified partners of SOCS proteins. Exhaustive studies are presently being performed to elucidate the molecular mechanisms behind the inhibitory action of SOCS proteins.

1.4.2.1.2 Growth Hormone Signaling and SOCS proteins

GH induces SOCS expression with different kinetics in F442A fibroblast (285), several tissues in hypophysectomized animals (286) and primary hepatocytes (287). Generally, CIS and SOCS3 expression is rapidly induced after GH stimulation, but transient, while SOCS2 expression increases steadily with time, being maximal 24h after stimulation.

SOCS1 and SOCS3 overexpression provokes a total inhibition of GH-dependent signaling (phosphorylation of STAT5 and JAK2, DNA-binding capacity of STAT5 and GH-dependent reporter activity) while SOCS2 and CIS have only a partial effect (265, 285, 288). It has been proposed that SOCS can inhibit GH signaling through its interaction with the activated receptor and/or JAK2. This would affect the binding of other signaling molecules and probably induce GHR/JAK2 degradation. SOCS1 binds

JAK2 with high affinity and induces its degradation (277) while the other SOCS proteins bind the GHR directly. SOCS3 binds to the phosphorylated Y487 and Y332 (265) while SOCS2 uses Y595 (253). It seems however, that SOCS1 also binds to GHR but in this case, receptor activation is not required (265, 288). CIS inhibitory action seems to be time-dependent and to involve direct binding to GHR and the induction of GHR proteasome-dependent degradation (227, 281). The specific sites on the GHR required for the interaction with CIS have not been mapped yet. It is important to mention that CIS overexpression (transgenic mice) results in a phenotype similar to the one found in mice lacking STAT5b. This suggests that CIS may be an important player in the downregulation of GH-dependent signaling.

The phenotype found in SOCS2 deficient mice ($SOCS2^{-/-}$), identifies SOCS2 as a main player in the negative regulation of GH signaling. $SOCS2^{-/-}$ mice are 30-40% larger that their littermates (254). The weight gain is due to an increase in bone size and a proportionate enlargement of most organs. Similar phenotypes have been also found in animals overexpressing GH (289), acromegalic patients (32) and a spontaneous mutation in mice that causes 40-50% increase in postnatal growth denominated High-Growth (hg). It has been established that hg mice have a deletion within the chromosome 10 resulting in a disruption and inactivation of the *socs2* locus (290).

IGF-1 mRNA expression in $SOCS2^{-/-}$ is significantly increased in some organs, without major changes in hepatic IGF-1 content. Interestingly, the GH-dependent gene product MUP was decreased in these animals, suggesting a GH overactivity (291). Furthermore, primary hepatocytes derived from these animals have a mildly prolonged STAT5 activation in response to GH. The necessity of STAT5b for the gigantism observed in $SOCS2^{-/-}$ mice was demonstrated by the generation of double knockout mice lacking both genes (292). On the other hand, the generation of SOCS2 transgenic mice has given surprising results. Instead of the expected growth inhibition, these mice show a 10% increase in body growth (253). SOCS2 is able to bind the endogenous GHR through the Y595 residue in several organs, including liver, muscle and lung (253). SOCS2 can also bind IGF-1 receptors (267). *In vitro* experiments also show that SOCS2 have dual effects on GH actions. By transfection of SOCS2 expression vectors in cell lines, it was observed that high concentrations of SOCS2 have an enhancing role instead of an inhibitory action on GH signaling (285). Forced overexpression of SOCS2 in mice and cell cultures may however result in very high expression levels, leading to cellular responses that are difficult to interpret. These results have to be further explored to demonstrate that the concentration when SOCS2 has stimulatory actions can be achieved in physiological situations.

Further studies have demonstrated that indeed, SOCS2 is essential to downregulate GH signaling. For example, SOCS2 blocks GH-dependent inhibition of neural stem cells differentiation. Consequently $SOCS2^{-/-}$ mice have less neurons in the developing cortex, while SOCS2 overexpression results in increased neural differentiation (293). It has also been shown that inhibitory action of estrogen on GH signaling is mediated by SOCS2. Estrogen increases SOCS2 mRNA, which then suppresses GH-dependent

JAK2 phosphorylation (294). Recently, Miller and co-workers demonstrated that SOCS2 inhibits intestinal epithelial cell proliferation, which is induced by GH and IGF-1 (295).

These findings provide strong indications that the GH/IGF-1 axis is a main target for SOCS2 actions. However, the exact mechanisms whereby each of the SOCS proteins, in particular SOCS2, exerts their negative actions on GH signaling are not fully understood. The figure 10 summarizes the mode of action of SOCS proteins on GH signaling.

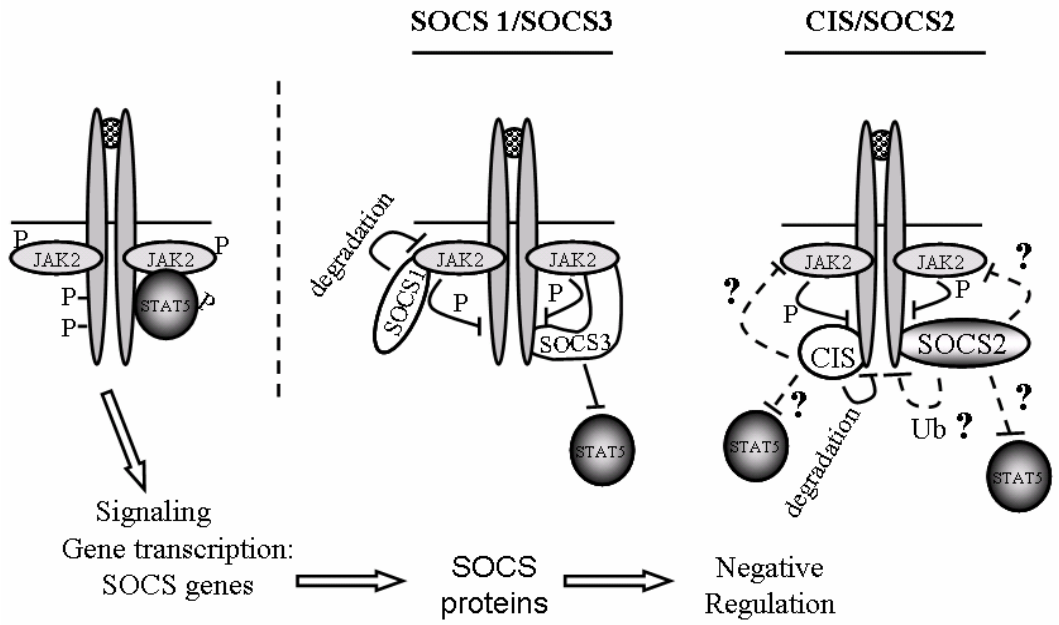


Figure 10. Mode of action of SOCS proteins on GH signaling. Once the GH-dependent JAK2/STAT5 signaling is activated, transcription and translation of SOCS proteins is increased. Then, SOCS proteins turn off signaling through binding to GHR and/or JAK2, blocking the access of other signaling proteins as STAT and/or inducing the degradation of GHR or JAK2.

1.4.2.2 Phosphatases

Activation of GH-dependent signaling pathways is based on protein phosphorylation on tyrosine, serine or threonine residues. The obvious mechanism for deactivation of this process is the action of protein phosphatases. Indeed, it has been shown that pre-incubation with Vanadate (pervanadate or ortovanadate), which is a general tyrosine phosphatases inhibitor, induces the prolongation of GH-dependent JAK2 and STAT5 phosphorylation (296). Considering that the GHR/JAK2/STAT5 cascade is regarded as the main GH-activated signaling pathway, the action of phosphatases on these three proteins is critical for the cellular response.

Several studies have identified three different phosphatases, (i) SHP1 (SH2 domain-containing protein-tyrosine phosphatase 1, also known as PTP-1) (ii) PTP1b and (iii) PTP-H1 to be involved in the specific down regulation of GH effects. GH can activate

SHP1 and induce its translocation to the nucleus where it binds to phosphorylated STAT5b (297), resulting in an attenuation of STAT5 activity. SHP1 also binds GH-activated JAK2 in FDP-C1 cells and controls the duration of GH-dependent JAK2 phosphorylation in the liver. Hepatic GH signaling is prolonged in mice lacking SHP1 (298).

Besides SHP1, PTP1b can also regulate GH signaling. A recent study by Gu *et. al.,* (299) has shown that PTP1b interacts with JAK2 in a GH-dependent way, and dephosphorylates the tyrosines present in the active JAK2 molecule. Fibroblasts derived from mice lacking PTP1b show JAK2 hyperphosphorylation and an enhancement of STAT3 and STAT5 phosphorylation upon GH treatment. Overexpression of PTP1b reduces GH-dependent ALS promoter activity in H4IIE cells. Two other phosphatases, TC-PTP and PTP-PEST were tested in the same system, without any effect on the reporter activity. Experiments *in vivo* indicate that absence of PTP1b reverses the hepatic GH resistance that is observed in some stress conditions such as fasting.

Pasquali and co-workers (300) have identified four protein tyrosine phosphatases (PTP) with binding activity towards the GH-induced phosphorylated GHR. Interestingly, one of these phosphatases is PTP1b, which was previously shown to regulate GH-dependent signaling and gene expression. The others are PTP-H1, SAP-1 (stomach cancer-associated PTP1) and TC-PTP. TC-PTP has, however, no effect on GH-dependent reporter activity according to the study described above (299). By over-expression of wild type and mutant variants of each phosphatase, it was shown that PTP1b and PTP-H1 can dephosphorylate the GHR. The mRNA expression in two GH-responsive tissues shows that TC-PTP and PTP-H1 levels are 3-4 times higher than PTP1b levels in the liver while only the expression of PTP-H1 and PTP1b was detected in muscle. It is worth noticing that GHR dephosphorylation in these cellular models is not total, suggesting that there are other phosphatases or that different mechanisms are required for the complete silencing of GH-dependent signaling.

1.4.2.3 Signal Regulatory Protein α (SIRP-α)

SIRP-α belongs to a family of ubiquitously expressed transmembrane glycoproteins, which also includes SIRP-β members. Structurally, SIRP-β members lack the cytoplasmic region present in SIRP-α while sharing 90% homology in the extracellular domain (301). SIRP-α was identified by their ability to associate to the SH2 domain of SHP-2, SHP-1 and Grb2 in response to Insulin, EGF and PDGF. The binding to SHP-2 is mediated by tyrosine phosphorylated residues in SIRP-α. The growth response to insulin and EGF in NIH3T3 cells is inhibited by SIRP-α through a reduction in the activation of MAP kinase pathway (302).

GH induces JAK2-dependent phosphorylation of SIRP-α and its ability to bind SHP2 (303). Overexpression of SIRP-α negatively regulates GH-activated signaling by inhibition of the phosphorylation of JAK2, STAT5b, STAT3, ERK1 and ERK2. This effect is not observed when a SIRP 4YF (a mutant lacking four cytoplasmic tyrosines)

is overexpressed, further confirming the negative action of SIRP-α on GH signaling (304). The exact mechanism whereby SIRP-α negatively controls GH signaling is not clear. It can involve binding to SHP-1, a protein tyrosine phosphatase known to act on JAK2 and STAT5 (297, 298). Alternatively, it could compete with the GHR for binding of positive regulators such as SHP-2 (305) or JAK2.

1.4.2.4 Protein Inhibitors of Activated STATs (PIAS)

PIAS are constitutively expressed proteins that were identified as negative regulators of cytokine signaling because of their capacity to inhibit the activity of STAT-transcription factors. There is also evidence indicating that PIAS can act as transcriptional co-regulators in other signaling pathways such as those use by steroid hormone and p53. More recent evidence indicates that PIAS can act as E3-like ligases stimulating the binding of ubiquitin-like SUMO modifiers to target proteins (306, 307).

Six PIAS have been identified; PIAS1, PIAS3, PIAS3β, PIASxα, PIASxβ, PIASy,) which are able to interact with different transcription factors and other partner proteins. The following table shows interactions of GH-regulated transcription factors and members of PIAS family.

Table 9. Actions of PIAS on known GH-activated transcription factors (TF)

TF	***PIAS***	***Functional Role of PIAS***
STAT1	PIAS1	Inhibition of DNA-binding activity resulting in inhibition of STAT1-mediated gene activation.
	PIASy	Transcriptional co-repressor
STAT3	PIAS3	Blocking of DNA-binding activity resulting in inhibition of STAT3-mediated gene activation.
STAT5	PIAS3	Inhibition of DNA-binding activity and repression of STAT5 transcriptional activity.
IRF-1	PIAS3	Inhibition of transactivation
C/EBPα	PIASy	Inhibition of transactivation
c-Jun	PIAS1	Unknown
	PIASx	Unknown

For references, see Schmidt and Müller (307).

As it is shown in the table, the only interaction between STAT5 and a member of the PIAS family is with PIAS3. Nevertheless, there is no record regarding a modulatory action of any of the PIAS on GH-dependent STAT5, STAT3 or STAT1 activation. It is important to mention that several new GH-activated transcription factors have been recently identified which are potential targets for the action of PIAS. Therefore a regulatory role for this family on certain GH actions cannot be excluded.

1.4.3 Summary

The capacity of a cell to respond to GH is tightly regulated. The magnitude and the duration of the signal is limited and affected by the intracellular environment. Various mechanisms of cross-talk exist with signaling pathways that can negatively influence

GH responses. An impaired down regulation of GH signaling can result in uncontrolled responses to the hormone affecting the normal homeostasis and leading to different pathophysiological complications. Conditions such as GH hyperactivation, GH-resistance and probably some metabolic disorders like diabetes, may be related to an impaired negative regulation on GH signaling. Although there are several mechanisms whereby GH signaling can be turned off (Figure 11), the phenotype found in SOCS2-deficient mice ($SOCS2^{-/-}$) indicates that SOCS2 is a main player in the negative regulation of GH signaling. The action of SOCS proteins, as described above, can be connected to the internalization process through the regulation of GHR ubiquitination, although the mechanistic details behind this process are not yet known. In this thesis we explore some of the mechanisms that negatively regulate GH signaling.

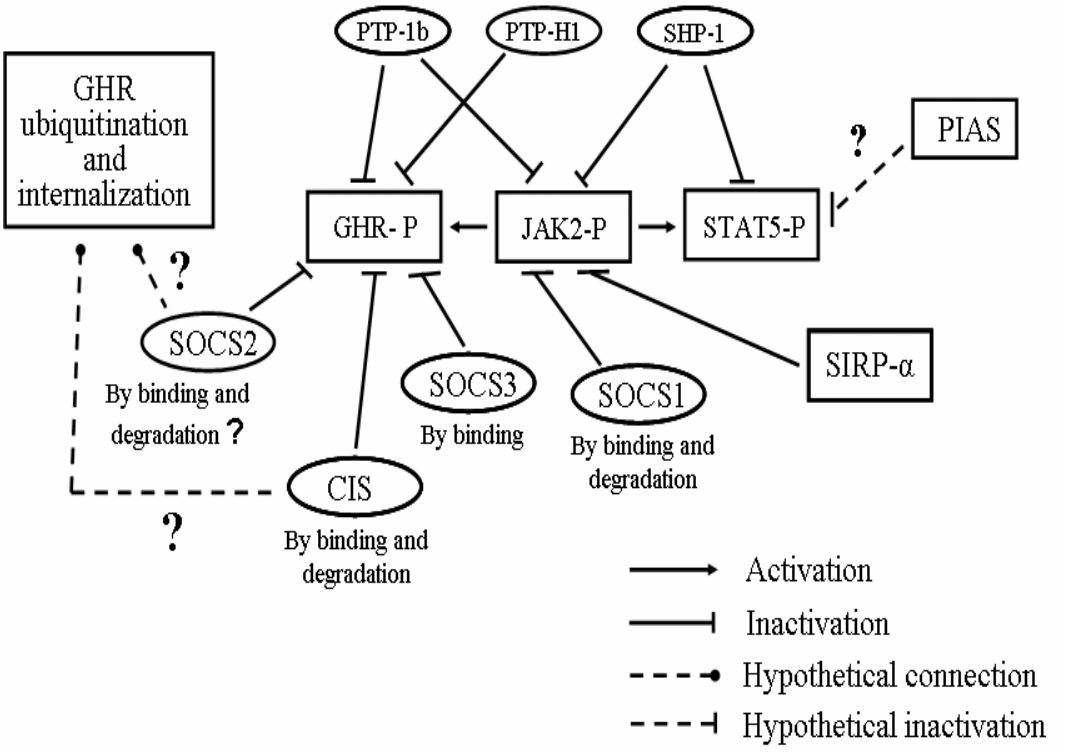


Figure 11. Negative regulation of GH signaling. The figure shows the post-receptor events that can negatively regulate GH-dependent signaling. The ubiquitination and internalization processes are required for the negative regulation of GHR activity.

1.5 AIMS OF THE STUDY:

1.5.1 General

The overall aim of this thesis was to identify pathways that affect GH sensitivity in target tissues and to evaluate the role of SOCS proteins as negative regulators of GH signaling.

1.5.2 Specific

1. To study a putative interaction between endoplasmic reticulum stress or actin cytoskeleton polymerization and the GH-activated JAK2/STAT5 pathway.

2. To characterize the SOCS2$^{-/-}$ giant mice in terms of hepatic gene expression and metabolic parameters.

3. To determine whether the giant phenotype of SOCS2$^{-/-}$ mice requires GH.

4. To study the molecular mechanisms of SOCS2 action *in vitro*, including mutagenesis analysis of SOCS2 protein domains.

1.6 MATERIALS AND METHODS

1.6.1 Materials:

1.6.1.1 Cell lines (Papers I, II and IV)

In papers I and II, a liver-derived cell line was used as biological model. Buffalo rat liver cells stably transfected with the rat GHR (BRL-4) have been characterized previously (308). BRL-4 cells have been shown to respond to GH according to several criterias, including the activation of JAK2/STAT5 pathway (175).

In paper IV, the human embryonic kidney cell line 293T was used to study the actions of SOCS2 and SOCS2 mutants on GH-dependent reporter activity and to evaluate SOCS2 and Elongin BC interactions. In these experiments, cells were transfected with a constant amount of GHR-plasmid and variable concentrations of SOCS2 plasmid.

1.6.1.2 Animal Models (Papers III and IV)

In paper III, several tissues from SOCS2-deficient mice ($SOCS2^{-/-}$) were used as biological material. $SOCS2^{-/-}$ mice were generated in The Walter and Eliza Hall Institute of Health (Melbourne, Australia) and some of their phenotypic features have been previously published (254). Gene expression was measured by microarray analysis or real-time RT-PCR.

In paper IV, double knockout mice, denominated $SOCS2^{-/-}Ghrhr^{lit/lit}$, were used to study the role of SOCS2 in the regulation of GH actions. $Ghrhr^{lit/lit}$ mice have a point mutation in the GHRH receptor that causes a near complete deficiency in circulating GH, resulting in dwarfism (309). Double mutated mice were generated by mating $SOCS2^{-/-}$ with $Ghrhr^{lit/lit}$ mice. Heterozygous were identified in the F1 generation. SOCS2 deletion was detected by genotyping as described in Metcalf *et. al.*, (254). Genotyping of $Ghrhr^{lit/lit}$ mutation was performed by PCR across the mutation followed by direct sequencing of PCR products (310). Second matings between male $SOCS2^{-/-}Ghrhr^{lit/lit}$ or $SOCS2^{-/-}Ghrhr^{lit/wt}$ with female $SOCS2^{-/-}Ghrhr^{lit/lit}$ or $SOCS2^{-/-}Ghrhr^{lit/wt}$ were performed to establish the colony.

1.6.2 Methods:

In papers I and II common techniques in cell biology were used to study the down-regulation of GH signaling. Methods such as RNAse Protection Assay, Immunoprecipitation, Western Blot, Gel Electrophoretic Mobility Shift Assay (GEMSA), Metabolic Labelling, Internalization assay and transient transfections were widely used and are fully described in papers I to IV.

1.6.2.1 Gene expression analysis (Papers III and IV)

DNA microarray allows the analysis of gene expression changes of thousands of genes in a single assay. Application of microarray technology in the endocrine field is rapidly expanding our knowledge on hormones molecular actions.

DNA microarrays consist of DNA probes immobilized onto a non-porous surface (matrix) in an ordered fashion, at high density. Microarray analysis is a hybridization based method. RNA species from two samples (control and testing) are transformed to cDNA by reverse transcription while fluorescently labeled. The samples are then allowed to hybridize onto the matrix. By measuring the amount of cDNA bound to each spot (fluorescence intensity), it is possible to determine how much the expression of a particular gene changes with the treatment.

The main forms of gene chips available are the cDNA and oligonucleotide microarrays. cDNA microarray probes are prepared by PCR amplification from cDNA clones, representing expressed sequence tags (EST) and known genes, and robotically spotted onto glass slides. Oligonucleotide arrays that use longer oligonucleotide (60-70 mers) probes can also be fabricated in a similar way. Arrays of short oligonucleotides (25 oligomers) made by *in situ* photolithography are commercially available from Affymetrix, Inc.

In this thesis, we have used microarray analysis as a tool to further explore the actions of SOCS2. In paper III, we use cDNA microarrays to characterize the hepatic gene expression profile in absence of SOCS2. The fabrication of the cDNA microarray has been described previously (57). It contains approximately 6200 rat/mouse cDNA probes from TIGR Gene Index, Research Genetics and our own probe collection. In paper IV, a mouse oligonucleotide microarray containing 16500 mouse oligo probes (70 mers) produced at the SweGene DNA Microarray resource center, Lund University (Sweden) was used to demonstrate the hepatic overreactivity to GH treatment in mice lacking SOCS2.

1.6.2.1.1 Target preparation and hybridization

In paper III, total hepatic RNA (25 μg) was extracted from 4 control (wild-type mice) and 4 test samples ($SOCS2^{-/-}$ mice) and individually labeled with different fluorescent dyes (cyanine-3 (Cy3) and cyanine-5 (Cy5)). Since the efficiency of the labeling is not even for the two dyes, a dye-swap design in the labeling of the samples was included. In paper IV, twelve independent hybridisations were performed comparing 4 individual animals from 3 different experimental groups ($SOCS2^{+/+}Ghrhr^{lit/lit}$ vs $SOCS2^{-/-}Ghrhr^{lit/lit}$, $SOCS2^{+/+}Ghrhr^{lit/lit}$ vs $SOCS2^{+/+}Ghrhr^{lit/lit}$ + GH and $SOCS2^{-/-}Ghrhr^{lit/lit}$ vs $SOCS2^{-/-}Ghrhr^{lit/lit}$ + GH). The protocols for labeling and hybridization have already been described (57, 311).

1.6.2.1.2 Data collection, normalization and analysis

Arrays were scanned using a confocal laser (Affymetrix, CA) capable of simultaneously detecting the emission from the two dyes. The laser intensity and detector gain were adjusted to yield images with non-saturated spots. We used GenePix Pro software (Axon Instruments, CA) to calculate the relative expression levels of each gene. After spot identification, the background-substrated hybridization intensities are calculated for each spot and a first transformation of the data (logarithmic conversion of

the expression ratios) was performed, followed by data normalization. Normalization is necessary to eliminate artifacts caused by unequal quantities of RNA input and differences in labeling and detection efficiencies between the dyes. In this thesis we have used the local weighted linear regression (Lowess) method, which assumes that the majority of the genes do not differ between the samples (312). The Lowess algorithm performs a local fit to the data in an intensity-dependent manner. The intensity value for each spot is normalized based on data distribution in the immediate neighborhood of the spot's intensity.

After normalization, the raw data is further statistically analyzed in order to identify genes that are differentially expressed between the two samples. Permutations tests, carried out by repeatedly scrambling the samples class labels and computing t statistics for all genes in the scrambles data, are the best tests to capture the structure of the data. In papers III and IV we used the SAM (Significance Analysis of Microarrays) statistical technique especially designed for microarray analysis (313). This method assigns a score to each transcript based on its change in gene expression in relation to the standard deviation of replicated measurements for that particular gene. SAM also estimates the false discovery rate (FDR), which is the percentage of genes expected to be wrongly identified as differentially expressed. Each gene is assigned a q-value that indicates the minimum FDR at which the differential expression of the gene is called significant. In the paper III, genes with a FDR value less than 12% were listed as differentially expressed. In paper IV, a FDR of less than 5% was used to identify differential expressed genes. A further requirement was added to the statistical criteria to correct for differential expression based on the absolute changes in gene expression ratios. A value of 1.5 (50%) was chosen to denote differences (increased or decreased expression) in the level of hybridization between control and the sample of interest. Although lower statistically significant levels of changes in gene expression may have important biological consequences, insufficient information exists regarding its reproducibility by independent methodologies.

1.6.2.1.3 Hierarchical cluster analysis of gene expression profiles.

Cluster analysis is a very useful unsupervised method to organize multivariate data into groups with roughly similar behavior. It can be applied to a group of genes or to a collection of different experiments. One of the most common methods used in relation to microarray data is hierarchical clustering. The principle is to calculate all pairwise distances and to find the two genes (or experiments) that are more closely located in the n-dimensional space. These two genes are then grouped together and treated as one. The process is repeated until all the genes have been grouped. Different metrics can be used to calculate the distance between group of genes (or group of experiments). Euclidean distance takes into account the magnitude in the differences in expression. Pearson correlation coefficient evaluates trends of expression over a set of conditions when the magnitude of the changes is not of importance (314).

In the paper III, we used average-linkage hierarchical clustering with the Pearson correlation coefficient, as a measurement of similarity between different groups of

experiments (315). While this methodology has been successfully used in numerous studies to discover mechanistic relationships between different biological samples, there are limitations to this approach. They arise from the use of different experimental models and technical platforms as well as from limitations in the expression profiles available for analysis. To minimize those effects we selected experiments from our own database (www.cmm.ki.se/EndoGED) and public data from Gene Expression Omnibus (GEO) (available at http://www.ncbi.nlm.nih.gov/geo/) that bear relevance/similarity to the phenotype of $SOCS2^{-/-}$ mice. In order to compare the different platforms, orthologues probes in both arrays were identified using the TIGR Orthologue Gene Alignment Database (TOGA) as implemented in RESOURCERER (316); a software for annotating and linking microarray resources within and across species (available at http://www.tigr.org/tigr-scripts/magic/r1.pl.). Cluster analysis was performed using the TIGR Multiple Experimental Array Viewer (http://www.tigr.org).

The experiments used in the generation of the expression matrix and the clustering analysis in paper III are described in detail in the respective publication or in the web page www.cmm.ki.se/EndoGED. The complete list of significantly regulated genes obtained in the studies in paper III and IV is available at www.cmm.ki.se/EndoGED.

1.6.2.2 Quantitative Real-time RT-PCR (Paper III and IV)

Quantitative real-time RT-PCR was used to verify some of the results obtained by microarray analysis. We used a non-sequence specific fluorescent dye SYBR Green I, which specifically incorporates into the double stranded DNA, to measure the amplicon concentration at the end of each PCR cycle. Since this method does not need a fluorescence-labelled oligonucleotide probe, it provides an economic and easy way to quantify mRNA. However, the disadvantage is that the dyes bind to any double stranded DNA being incapable to distinguish between the PCR product of interest and alternative products such as primer-dimers. This problem is avoided by the prior validation of the primers and also by generating a melting curve of the amplicon. The RNA species can be quantified using either absolute or relative quantification. The absolute method determines the amount of target, expressed as copy number or concentration. The relative quantification determines the ratio between the amount of target and a housekeeping gene with constant expression levels in the samples that are compared. The later method was used in papers III and IV. The primers were designed using the Primer 3 program available at: *http://www.broad.mit.edu/cgi-bin/primer /primer3_www.cgi*. In all cases, β-actin was used as housekeeping gene.

1.6.2.3 BIAcore (Paper IV)

A BIAcore is an optical device that can measure real time changes in refractive index near a planar surface. This biosensor-based technology employs surface plasmon resonance (SPR) for label-free measurements studies on molecular interactions (317). The SPR system combined with the miniaturized flow system provides efficient sample and buffer delivery to the sensor surfaces and permits continuous monitoring of complex formation and the dissociation of analyte from immobilized ligand (318). The

output signal of a BIAcore is measured in resonance units (RU). The instrument consists of the optical interface where the chip (with a carboxy-methylated dextran surface) is mounted, and the microfluidic system that allows the interaction between the sample and the chip.

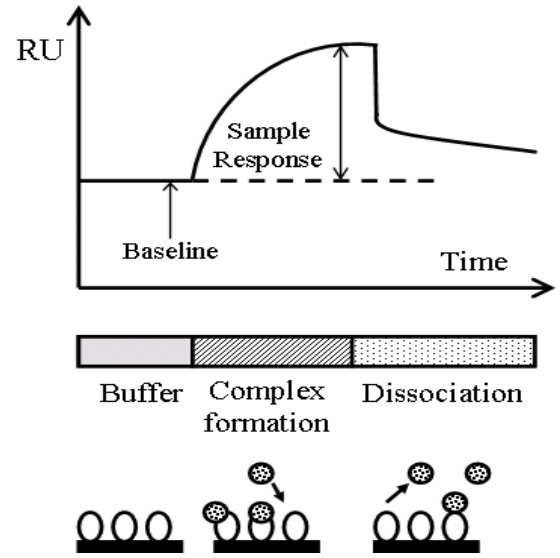


Figure 12. Representation of the sensorgram and the events related to the BIAcore detection. Adapted from Karlsson (318).

In paper IV we have used BIAcore analysis to demonstrate the interaction between SOCS2 and the phosphorylated GHR. Biotinylated phosphopeptides derived from the GHR were fixed to the biosensor chip (SA5, Biacore, Sweden) and the recombinant SOCS2 SH2 was passed over the chip allowing the measurement of protein interactions.

1.6.2.4 AlphaScreen™ Binding Assay (Amplified Luminescent Proximity Homogeneous Assay) (Paper IV)

Measurement of protein-protein interactions by standard methods usually requires a physical separation of the protein-protein complex and the unbound proteins. With this technology the separation step is not necessary since the signal is modulated by the molecular binding event in real-time. This technique, originally known as LOCI (Luminescent Oxygen Channeling Immunoassay), is relatively insensitive to interference by particles or other substances present in the biological samples (319, 320). The method utilizes the aggregation of particles containing-proteins (particle-particle interactions) where the donor particle contains a photosensitizer (phthalocyanine) while the acceptor particle contains a chemiluminescer (thioxene derivaties). Irradiation of the donor particle at 680 nm causes photosensitized formation of singlet oxygen, which diffuses approximately 200 nm in solution. If the acceptor bead is within that distance, energy is transferred from the singlet oxygen to the chemiluminiscencer within the acceptor, culminating in light emission at 520-620 nm (www.perkimelmer.com/lifesciences).

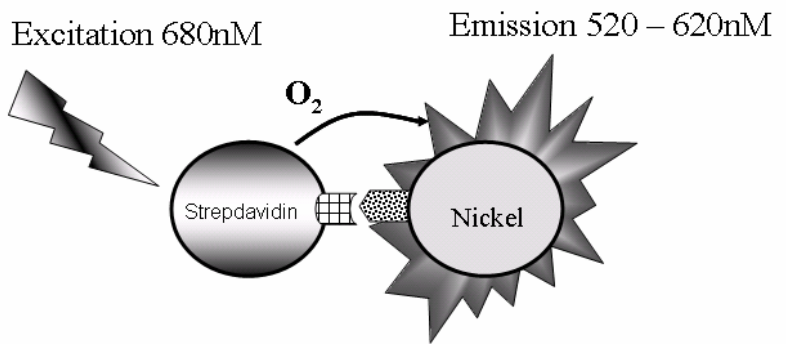


Figure 13. Representation of the events related to AlphaScreen Binding Assay. Adapted from PerkimElmer ® (www.perkimelmer.com/lifesciences).

In paper IV, we used nickel chelate derivatised Alpha Screen™ beads to demonstrate the interaction of SOCS2 and different phosphopeptides derived from GHR. The donor beads (streptavidin-coated) were incubated with the biotinylated phosphopeptides allowing the coating of the bead. The acceptor beads were coated with recombinant SOCS2-SH2 domain. The interaction between the particles was promoted by incubation of the donor and acceptor beads at room temperature for 2.5h.

1.7 RESULTS

1.7.1 Pathways that affect GH sensitivity in the liver (Paper I and II)

Many pathological situations are accompanied by induction of cellular stress. The cell reacts to those stressful situations through the activation of pathways designed to restore homeostasis or alternatively, induce apoptosis. The cellular response depends on the nature of the stress and relies on the presence of specific sensors that can distinguish between different types of stress. The endoplasmic reticulum (ER) is the quality control site for accurate folding of secreted and membrane proteins. The unfolded protein response (UPR) is activated by the accumulation of misfolded proteins in the ER. It consists of a general inhibition of "*de novo*" protein synthesis, a selective activation of ER chaperones and an active transport of misfolded proteins outside the ER for proteasomal degradation (321). The UPR is especially important to control the homeostasis of tissues with significant secretory functions, such as the liver. In intact organisms the UPR is not an isolated phenomenon but occurs concomitantly with the actions of cytokines, growth factors and hormones. Since the JAK/STAT pathway regulates important cellular functions, it seemed relevant to investigate the relation between cytokine activated signaling pathways and the UPR. As a test system, we used the JAK2/STAT5 pathway induced by GH.

Cell experiments were carried out to investigate GH-activated signaling during BAPTA-AM-induced ER stress. BAPTA-AM (an intracellular calcium chelator) induces ER stress, blocks protein translation and activates the UPR. We observed that GH by itself causes a rapid induction of the transcription factor and stress regulator: C/EBP homology protein (CHOP), indicating a regulatory role of GH on the ER stress. The initial GH-dependent activation of the JAK2/STAT5 pathway was not affected by the UPR. However, we showed that the UPR extends the duration of the JAK2/STAT5 signaling pathway, modulating the rate of JAK2 and STAT5 dephosphorylation and STAT5 DNA-binding activity. Two other ER stressors, DTT (a reducing agent) and A23187 (a calcium ionophore), were also tested. These compounds also resulted in a prolonged GH signal. Since the SOCS proteins are involved in the negative regulation of GH signaling, we investigated the effect of ER stress on SOCS expression at mRNA and protein levels. According to our results the UPR response to ER stress does not inhibit the effects of GH on SOCS expression, except for a small reduction in the amount of SOCS2 2h post stimuli. These results suggest that cellular stress modulates the down regulation of JAK2/STAT5 pathway through mechanisms other than the inhibition of SOCS protein synthesis.

Results obtained after the publication of the paper indicate that the induction of ER stress regulates the levels of GHR ubiquitination. Figure 14 shows that treatment of the cells with BAPTA decreases the degree of GH-induced ubiquitination of the GHR, being more evident 1 and 2h after GH stimulation (lanes 5 and 7).

A decreased ubiquitination of the GHR may contribute to the prolongation of the GH-dependent JAK2/STAT5 activation evoked by ER stress, since proteasomal

degradation of the GHR would be reduced. The mechanism whereby the UPR decreases GHR ubiquitination is not clear, but it could be due to a relative deficiency in the amount of ubiquitin or ubiquitin ligases as a result of increased ubiquitination of misfolded proteins at the ER.

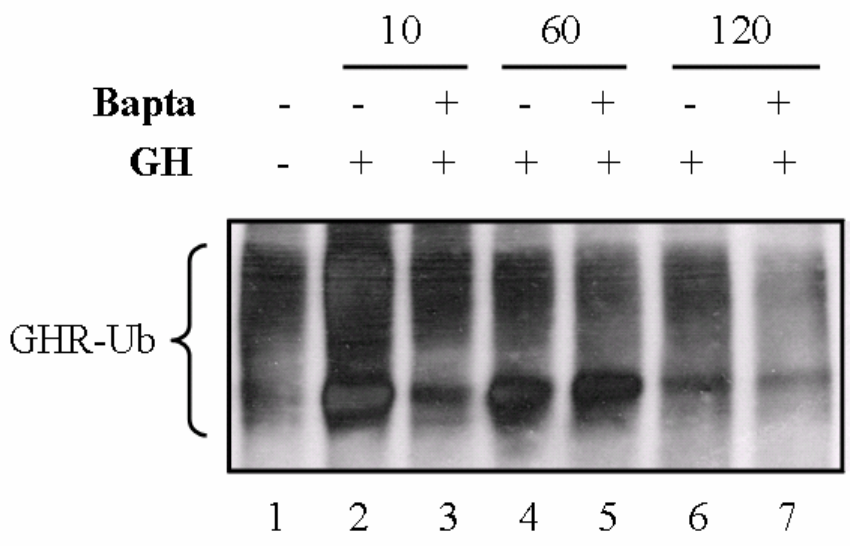
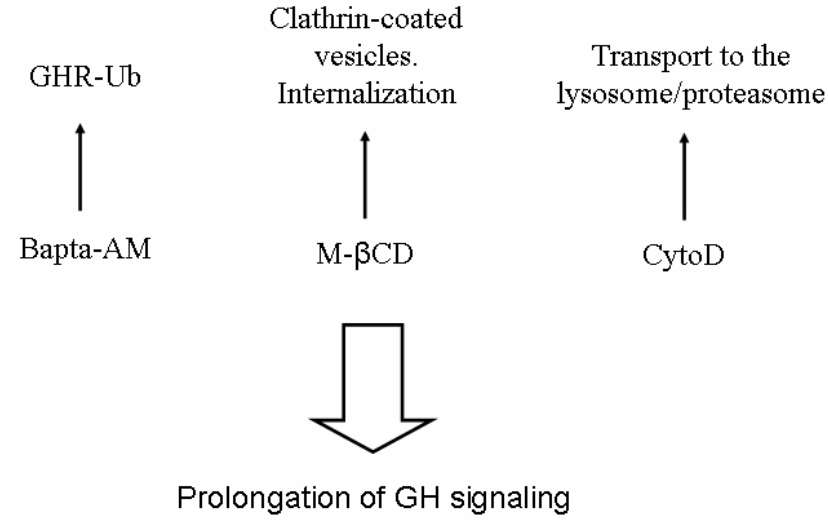


Figure 14. Ubiquitination of the GHR in presence of endoplasmic reticulum stress. BRL-4 cells were incubated with BAPTA-AM (10μM) 30 minutes before GH induction (50 nM) in a time-course model. Total cell extracts were immunoprecipitated using an anti-GHR antibody and then western blot was performed using an anti-ubiquitin antibody.

In paper II, we continued to explore cellular pathways that influence the GHR signal. It has previously been demonstrated that the actin cytoskeleton is a key component in the downregulation of some membrane receptors (322) and that GH induces a rearrangement of actin cytoskeleton (203), microtubule polymerization (323), phosphorylation of FAK (138) and cell motility (141, 324, 325). It is also known that an intact cytoskeleton is necessary for the internalization of some membrane receptors (322, 326), the uptake of ligands (327), the trafficking of molecules within the cell (328) and in general the correct functioning of the endocytic pathway (329). In this study we demonstrated that the integrity of the actin cytoskeleton is in fact a key component in the down regulation of GH signaling. Our data demonstrate that while the GH-induced activation of JAK2 and STAT5 does not require an intact cytoskeleton, the subsequent desensitization does. JAK2 tyrosine phosphorylation as well as STAT5 tyrosine phosphorylation and DNA binding activity are prolonged in the presence of cytochalasin D (CytoD), an inhibitor of the actin polymerization. Furthermore, the disruption of the cytoskeleton increases GH-dependent reporter gene activity, indicating that the down-regulation of GH-signaling depends on an intact actin cytoskeleton. Similar results regarding the phosphorylation of STAT5 were found when an actin filament-disrupting agent (Latrunculin A) was used. However, when an actin filament-stabilizing agent (Jasplakinolide) was tested, the deactivation of GH-dependent signaling was not affected. The treatment with CytoD did not affect SOCS1, 2 or 3 protein expressions.

Previous findings have demonstrated that inhibition of the proteasome prolongs GH-dependent signaling probably through inhibition of GHR internalization and accumulation of the ubiquitinated forms of the receptor at the cell membrane (209, 226). However, there was no evidence that inhibition of internalization also leads to an impaired down-regulation of the signal. We therefore decided to explore if internalization of the GHR influences the down regulation of the signal. When inhibition of the GHR internalization was blocked, using an inhibitor of the clathrin-coated vesicle formation (MβCD), we observed a prolongation of the GH-dependent STAT5 binding activity. This indicates that the internalization process is an important step in the negative regulation. Since GHR ubiquitination is needed for its turnover and the negative regulation of GH signaling, we analyzed if disruption of the integrity of the cytoskeleton affects GHR ubiquitination. CytoD treatment induces accumulation of the ubiquitinated GHR without affecting the internalization process, but it did inhibit degradation of the receptor. This indicates that the internalized GHR continues signaling while it is in the vesicles of the endocytic pathway, as has been suggested before (226). Our findings indicate that JAK2/STAT5-mediated transcription is potentiated by the cytoskeleton disruption most likely through inhibition of GHR degradation. The diagram shows a summary of the results obtained in papers I and II.



The first two papers demonstrate that the downregulation of GH signaling can be modulated by other intracellular pathways, specifically the UPR and those resulting in the actin cytoskeleton rearrangement. In both cases, regulation is exerted through mechanisms acting downstream SOCS1 and SOCS3 proteins or it could be independent of SOCS proteins. Another possibility could involve the action of SOCS2, probably mediating the degree of GHR ubiquitination and the process that follows.

1.7.2 Phenotypical characterization of SOCS2$^{-/-}$ mice (Paper III)

Several SOCS proteins act as negative regulators of GH signaling. However, SOCS mechanisms of action in relation to GH have been difficult to elucidate. By genetic elimination of individual SOCS genes, it was established that SOCS2 has a major role in regulating the GH/IGF-1 axis. These mice (SOCS2$^{-/-}$) show 30-40% increase in body

growth and some other characteristics found in GH overexpression models (254). We studied these mice to identify molecular characteristics that might contribute to their phenotype in relation to known GH actions.

The SOCS2$^{-/-}$ phenotype was analyzed using microarray expression profiling and the measurement of metabolic parameters. Our hypothesis was that the phenotype of these mice is a consequence of increased tissue sensitivity to GH. Using microarray and clustering analysis, we defined the hepatic gene expression profile in mice lacking SOCS2 and analyzed its similarity to other gene expression profiles relevant to GH actions. The general profile of SOCS2$^{-/-}$ has little similarity with other expression profiles where GH actions have been evaluated. Nevertheless, our results revealed that SOCS2$^{-/-}$ mice have indeed some molecular and metabolic changes that partially overlap with known GH-dependent actions. Some of the GH-like effects include an over expression of IGFBP3 (a well known GH-regulated gene) at the mRNA and protein levels, a decrease in serum triglyceride content and expression of lipoprotein lipase in adipose tissue. The increase in IGFBP3 is very interesting given some characteristics found in the IGFBP3 transgenic mice, such as the organomegaly affecting some organs (330). It has been established that IGFBP3 can have effects on cell proliferation that are independent of IGF-1 (331). IGFBP3 furthermore increases the half-life of circulating IGF-1 and the delivery of IGF-1 to target tissues. Even though SOCS2$^{-/-}$ mice do not have higher circulating levels of IGF-1, the overexpression of IGFBP3 could have a positive role in IGF-1 actions by increasing its half-life and thereby enhancing its actions on target tissues.

It is of interest that these mice do not show any anti-insulinic features as would have been expected from animals with hyperactive GH signaling. The glucose and insulin tolerance tests as well as the activation of hepatic insulin signaling are normal in these mice. The over expression of PGC-1α mRNA in skeletal muscle might contribute to the normal glycemic control observed in SOCS2-deficient mice. Alternatively IGFBP3 actions can promote the insulin-like effects of IGF-1.

Another important characteristic of SOCS2$^{-/-}$ mice is enlargement of their internal organs due to an increase in the number of cells rather than the size. From the gene expression profile, we show that absence of SOCS2 induces the over expression of genes that positively regulate cellular proliferation and the suppression of genes that negatively control this process, suggesting that SOCS2 might regulate other unidentified pathways.

In summary, our data indicate that SOCS2 deficiency is a "state of its own" partially sharing some features related to an increased GH activity, but also results in changes that cannot be related to known GH actions.

1.7.3 Role of SOCS2 in GH actions *in vivo* (Paper IV)

In this paper we tested the hypothesis that the phenotype of SOCS-deficient mice is dependent on GH action by studying whether GH deficiency would reverse the gigantic

phenotype of $SOCS2^{-/-}$ mice. By crossing $SOCS2^{-/-}$ mice with the $Ghrhr^{lit/lit}$ (GH-deficient) little mice, we showed that the gigantism observed in $SOCS2^{-/-}$ is indeed dependent on endogenous GH.

Male and female mice lacking both endogenous GH and SOCS2 showed impaired somatic growth resulting in dwarfism. Moreover, these mice are hypersensitive to GH, which was demonstrated by enhanced growth rate, increased weight of internal organs and hyperactivation of hepatic GH-dependent genes. The number of genes induced and repressed by GH as well as the expression ratios were significantly increased in the double knockout compared to the $SOCS2^{+/+}Ghrhr^{lit/lit}$ alone, indicating a hyperresponse to GH. This response was a general phenomenon that affected most genes, including well-known GH regulated genes such as IGF-1.

In this paper, we also demonstrated that three different domains of the SOCS2 protein have a crucial role for its action. By transient co-transfection of 293 cells, we showed that SOCS2 variants with a deletion of the N-terminal domain or the triple mutations in the SH2 domain failed to inhibit GH-dependent reporter activity. Deletion of the SOCS-box also blocked the inhibitory effect of SOCS2. Instead, elimination of the SOCS box enhanced the GH-dependent reporter activity. Since, it has previously been established that the SOCS-box of SOCS1 and SOCS3 can form complexes with Elongin BC (271, 272), we tested if a similar phenomenon was observed with SOCS2. As expected, SOCS2 co-immunoprecipitated with Elongin BC in 293T cells transfected with SOCS2. It was previously shown that SOCS2 binds the endogenous phosphorylated GHR in several tissues and that this interaction is mediated by the Y595 on the GHR. By BIAcore and Alpha Screen analysis we confirmed this previous finding and showed that an additional tyrosine residue on the GHR, Y487, is an important site of interaction. To demonstrate the importance of these interactions on GH-dependent signaling, tyrosine (Y595 and Y487) mutated GHR constructs were tested in GH-dependent reporter assays. Mutations of either Y595 or Y487 enhanced GH-dependent reporter activity and showed an additive effect when the double mutation was transfected. However, these effects did not reach statistical significance when the data was expressed as fold induction in comparison to unstimulated cells. Further analysis of the mutated GHR indicates that both residues are required for the inhibitory actions of SOCS2.

These results strongly suggest that SOCS2 is indeed an inhibitor of GH-activated signal transduction and that the mechanism of action involves binding to the phosphorylated GHR through the tyrosine 487 and 595 on the GHR and the SH2 domain on SOCS2. The N-terminal region and the SOCS-box of SOCS2 are important for its inhibitory actions and may involve the formation of a complex with Elongin BC. Since the SOCS box interacts with Elongin BC, it is possible that SOCS2 assemble an ubiquitin ligase complex that acts on the GHR/JAK2 complex.

1.8 GENERAL DISCUSSION

The activation phase of GH-dependent signaling pathways and their role in different GH actions have been extensively examined in previous studies. However, it has not been possible to establish the exact mechanism whereby GH signaling is turned off. Recent advances indicate that there are several interconnected processes that act in concert to turn off GH signaling. These include GHR ubiquitination, internalization and its degradation by the proteasome, and the action of negative regulators such as phosphatases and SOCS proteins.

In this thesis we have demonstrated that the GH-dependent JAK2/STAT5 signaling is modulated by UPR and actin cytoskeleton depolymerization. In these two cases, down regulation of the signaling is affected resulting in a prolonged phosphorylation of JAK2 and STAT5. However, the activation phase is not affected by these cellular pathways. Our results indicate that although both pathways induce a prolongation of the GH-dependent signal, the mechanisms behind these phenomena seem to differ and are reflected in the degree of ubiquitination of the receptor.

We demonstrated that UPR does not inhibit the mRNA expression of negative regulators such as SOCS proteins. On the contrary, the prolonged activation of JAK2/STAT5 in this condition induces a higher expression of SOCS genes. Since one characteristic of the UPR is to inhibit protein synthesis, we expected an inhibition of SOCS translation. Our results showed that levels of SOCS1 and SOCS3 proteins were barely affected by the UPR. However, SOCS2 was reduced 2h after induction of the UPR and this coincided with a reduction in the GHR ubiquitination (Figure 12). Reduction of GHR ubiquitination can prolong GH signaling through effects in the internalization process or in the proteolytic degradation of the receptor.

The mechanism whereby the UPR causes a reduction in GHR ubiquitination could be related to the availability of ubiquitin or ubiquitin ligases for the ubiquitination process. Unfolded proteins in the ER are transported outside the ER, ubiquitinated by specific ubiquitin ligases (Ubc6, Ubc7) (332) at the ER membrane and degraded by the proteasome. Accumulation of large amount of unfolded proteins can compromise the ubiquitination of proteins in the cytosol or the cell membrane (like the GHR). Another possibility is that SOCS2 is part of an ubiquitin ligase that acts on the GHR and that reduction in SOCS2 protein levels results in impaired ubiquitination of the GHR. In support of this, our findings demonstrated that SOCS2 binds the phosphorylated GHR through the Y487 and Y595, and inhibits the GH-dependent JAK2-STAT5 activation *in vitro*. Moreover, deletion of the SOCS box enhances GH-dependent reporter activity which indicates that the inhibitory action of SOCS2 requires the SOCS box domain. We also demonstrated that SOCS2 is able to bind Elongin BC, which is known to be part of other E3 ubiquitin ligase complexes. Data not shown in this thesis indicate that the overexpression of Elongin BC together with SOCS2 enhances the inhibitory action of SOCS2 on the GH-dependent reporter activity, further supporting the putative action of SOCS2 as an ubiquitin ligase.

Another mechanism whereby the UPR could interfere with the downregulation of the GHR signaling is the action of phosphatases on GH-activated signaling molecules. It has been determined that SHP1, PTP1b and PTP-H1 can dephosphorylate the components of GH-dependent JAK2/STAT5 pathway (298-300). Interestingly, PTP1b is mainly localized at the endoplasmic reticulum (ER) (333). PTP-1b is able to dephosphorylate EGFR and PDGFR on the surface of the ER after endocytosis of the receptors (334). It has also been shown that PTP1b is able to interact with JAK2 and dephosphorylate GHR and JAK2 (299, 300). Therefore the prolongation in the JAK2 phosphorylation associated to the UPR can be due to an impaired action of PTP1b on JAK2 at the ER caused by the ER stress. It is still unknown whether the endocytosis of the GHR/JAK2 activated complex precedes the action of PTP1b on GH signaling.

The ER is the cellular compartment in charge of synthesis and folding of secretory proteins and is consequently very important for the function of secretory organs such as liver (an important target for GH action). Our findings are the first demonstration of cross-talk between the UPR and cytokine-dependent signaling. GH is an important regulator of hepatic metabolic functions. In situations associated with ER stress such as exposure to drugs, xenobiotics or hypercholesterolemia (321, 335), a prolonged GH-dependent signal could modify GH actions in liver. It is known that GH administration has anti-insulinic action on hepatic glucose metabolism and also induces cholesterol and triglyceride synthesis. Therefore, a prolongation of the signal, caused by ER stress, could have deleterious effects. Clinical trials have demonstrated a dramatic improvement in mortality of critically ill surgery patients which are subject to significant stress by controlling glucose levels with insulin treatment (336). In contrast, GH administration to a similar group of patients dramatically increases mortality (337). Speculatively, this could be due to enhanced anti-insulinic actions of GH or other inflammatory cytokines due to stress. It will be interesting to explore GH and in general, cytokine sensitivity *in vivo* upon the induction of UPR since ER stress has been linked to several pathogenesis such as diabetes mellitus (338, 339), neurological diseases (321) and hypercholesterolemia (321, 335).

Our results indicate that depolymerization of the actin cytoskeleton, which neither affects the general protein translation nor the expression of SOCS proteins, also enhances GH-induced JAK2/STAT5 activity. This suggests that down regulation of the GH signaling occurs downstream or independent of SOCS proteins. Our findings that inhibition of clathrin-vesicle formation (one of the first steps in the endocytic pathway) prolongs GH signaling, indicates that GHR endocytosis/degradation is one of the mechanism that downregulate GHR signaling. Contrary to the case with the UPR, actin cytoskeleton depolymerization results in an accumulation of the different forms of ubiquitinated GHR. Interestingly, the internalization process was not blocked by the cytoskeleton depolymerization indicating that the ubiquitinated receptor accumulates upon internalization and remains active within endosomes, as has been demonstrated in other systems (226). The prolongation of the signal by CytoD might be due to an inhibition in the last steps of the endocytic pathway, including the transport to the lysosomes/proteasome and the degradation process. Alternatively, as the phosphatase

action of PTP1b seems to be exerted in the endoplasmic reticulum, it is possible that the disruption of the cytoskeleton affects the transport of the GHR/JAK2 complex to the vicinity of the ER, which could result in an inhibition of the dephosphorylation of the GHR and/or JAK2 (see diagram page 39).

Evidences from both *in vivo* and *in vitro* studies support the participation of SOCS2 in the downregulation of GH signaling. Elimination of the SOCS2 gene in mice induces a giant phenotype which resembles the one observed in GH overexpression models. $SOCS2^{-/-}$ mice do not have increased levels of circulating IGF-1 or GH but they show overexpression of IGF-1 mRNA in extrahepatic tissues and other characteristics that can be related to GH overactivity. By analyzing the response to GH of $SOCS2^{-/-}$ $GHRHR^{lit/lit}$ mice, we demonstrated that hyperresponsiveness to GH is the primary cause of the gigantism observed in $SOCS2^{-/-}$ mice, which agrees with the inhibitory action of SOCS2 on GH receptor. SOCS2 action on the GHR could be related to an ubiquitin ligase role as discussed before, but it could also act by blocking the access of positive regulators such as SHP2 (305) and/or STAT5, to the GHR.

Despite the fact that $SOCS2^{-/-}$ phenotype seems to arise from hypersensitivity of target tissues to GH stimulation, some of its phenotypic features differ from GH overexpression models. Similarities in hepatic gene expression profiles were limited to small number of genes such as IGFBP3 and SCoAD. Down regulation of Lpl in adipose tissue, increased liver triglycerides and reduction in circulating levels of triglycerides are features also observed in GH transgenic mice (340, 341). On the other hand, many characteristics of $SOCS2^{-/-}$ mice distinguish them from GH overexpression models. They have normal circulating levels of IGF-1, the glucose control mechanism seems unaltered and the liver shows regulation of genes that control cellular proliferation and that have not been shown to be GH regulated.

It is possible that the upregulation of few genes, which are also GH targets, has an important impact on certain aspects of the phenotype of these animals. For example, IGFBP3 could result in enhanced activity of IGF-1 with consequences both in somatic growth and glucose metabolism. Alternatively, it is possible that the distribution of SOCS2 expression differs from that of GHR, therefore causing hypersensitivity in selected tissues which in turn influences hepatic gene expression and the metabolic profile of these animals. Some of the effects caused by SOCS2 deletion in hepatic gene expression might be the result of other cytokine-dependent actions. In fact, the clustering analysis of gene expression profiles show that the $SOCS2^{-/-}$ profile has some similarity to gene expression profiles taken from the liver-specific $SOCS3^{-/-}$ and livers treated with IL-6. SOCS proteins also participate in the control of cytokine specificity as has been demonstrated in the liver specific deletion of SOCS3. IL-6 signaling in $SOCS3^{-/-}$ liver resembles INF-γ actions (255). A final possibility is that negative feedback mechanisms are also hyperactive, resulting in decreased secretion of GH. In fact a tendency to lower levels of GH is seen in $SOCS2^{-/-}$ mice (not statistically significant) (292). This bears similarity to the phenomena of long-term adaptation to

GH treatment in children and deficient adults when the initial treatment provokes severe diabetogenic effects that are reduced after long-term treatment (46).

In summary, the results presented in this thesis indicate that the duration of the GH signaling is controlled by signaling pathways such as the UPR and those that influence the actin cytoskeleton. These pathways mainly affect GHR ubiquitination suggesting an important role for ubiquitin ligases in the downregulation of GH signaling. The role of SOCS2 as a negative regulator for the GH signaling has been further validated by *in vivo* and *in vitro* studies. The mechanism of action of SOCS2 involves the binding to the GHR through the tyrosines 487 and 595 and the association with Elongin BC through its SOCS box. It is possible that the association of SOCS2 to Elongin BC serves to assemble an ubiquitin ligase that targets GHR/JAK2 to ubiquitination, thereby regulating internalization and possibly its degradation by the proteasome. The association of SOCS2 to the GHR could also block the access of positive regulators, such as STAT5 and SHP-2, to the GHR. Further studies are required to define the exact mechanism of action of SOCS2 and to evaluate the exiting hypotheses. Its is necessary to determine whether SOCS2 associates to ring finger proteins, cullins and E2 enzymes, which are the minimal components required to assemble a functional ubiquitin ligase. The final prove that SOCS2 is an ubiquitin ligase acting on GHR would require the demonstration that this activity can be reconstituted using purified components in cell free assays.

The timely degradation of receptors and signaling intermediaries offers novel opportunities for pharmaceutical intervention. The main component of this process seems to be exerted at the ubiquitination level, by highly specific ubiquitin ligase complexes. In this thesis, we have linked SOCS2 to the down regulation of GH signaling. These findings taken together with the demonstration that other pathways, in this case cellular stress, alter signaling by interferences with the silencing mechanism warrant further investigations. The characterization of SOCS proteins is especially important since, through their SH2 domain, they can target tyrosine phosphorylated proteins for degradation and theirs activities towards leptin, insulin and GH have been documented. Enhancing tissue sensitivity to metabolic hormones by inhibiting the proteolytic degradation of signaling molecules, offers an attractive possibility that radically differs from the current therapies. This is especially important in the treatment of insulin resistance where few therapeutic alternatives exist. The understanding of the mechanism how cytokines like GH induce insulin resistance may be highly relevant to design new therapies. For example, to use SOCS2 inhibitors to enhance the beneficial actions of endogenous GH in muscle and adipose tissue without the pitfall associated to direct GH treatment. In summary the study of the mechanisms of action of SOCS proteins may lead to improved therapeutics or diagnostics in areas of metabolic disorders where resistance to the actions of hormones like GH, leptin and insulin play a pathological role.

1.9 CONCLUSIONS

1.9.1 General

The studies presented in this thesis indicate that down regulation of GH signaling is a tightly controlled process, involving several regulatory steps. The negative regulation of GH signaling is modulated by stress in the endoplasmic reticulum, the actin cytoskeleton network, the rate of ubiquitination of the GHR and the action of SOCS proteins, particularly SOCS2.

1.9.2 Specific

1. Intracellular signaling pathways such as the unfolded protein response (UPR) and rearrangement of the actin cytoskeleton influence the down regulation of GH-activated JAK2/STAT5 signaling pathway in liver cells.

2. Ubiquitination of the GHR influences the duration of the GH-activated JAK/STAT signaling pathway.

3. Endogenous GH is required to develop the giant phenotype found in SOCS2-deficient mice indicating that SOCS2 is a negative regulator of GH signaling. Mice lacking GH and SOCS2 ($SOCS2^{-/-}Ghrgh^{lit/lit}$) are hyperresponsive to GH in terms of growth rate and hepatic gene expression.

4. The phenotype of SOCS2-deficient mice includes a distinct hepatic gene expression profile and metabolic features that are a mix of GH-like and GH-independent actions.

5. SOCS2 acts as a negative regulator of GH signaling *in vivo* and *in vitro*, by binding to the GHR and Elongin BC, probably acting as an ubiquitin ligase for the GHR.

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