

# **CIS/SOCS PROTEINS IN GROWTH HORMONE ACTION**

A Dissertation Presented

By

**LING DU**

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Science, Worcester  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

OCTOBER 2000

Molecular and Cellular Physiology

# **CIS/SOCS PROTEINS IN GROWTH HORMONE ACTION**

A Dissertation Presented

By

Ling Du

Approved as to style and content by:

(Signature) \_\_\_\_\_  
Julie Jonassen, Ph.D., Chairwoman of Committee

(Signature) \_\_\_\_\_  
Jack Leonard, Ph.D., Member of Committee

(Signature) \_\_\_\_\_  
Daniel Kilpatrick, Ph.D., Member of Committee

(Signature) \_\_\_\_\_  
Janet Stein, Ph.D., Member of Committee

(Signature) \_\_\_\_\_  
Jessica Schwartz, Ph.D., Member of Committee

(Signature) \_\_\_\_\_  
H.M. Goodman, Ph.D., Dissertation Mentor

(Signature) \_\_\_\_\_  
T.B. Miller, Jr., Ph.D., Dean of the Graduate  
School of Biomedical Sciences

Program of Molecular and Cellular Physiology

October, 2000

**DEDICATION**

*To my daughter, Emily (Shanshan), who brings joy to my life*

*To my husband, Xinzhong, who has been supporting me from all aspects,  
and whose encouragement and enthusiasm to science keep inspiring me*

*To my father who has been waiting for me home for the past seven years*

*and*

*To my mother who watches me from the heaven*

## ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my mentor, Dr. Goodman, who accepted me to his laboratory five years ago and provided me a scientific atmosphere of independence and creativity. His sincerity and ethics to science taught me what a real scientist should be. His optimism, encouragement and understanding helped me to look at achievements and failures objectively. I hope I made a fine graduate as his last graduate student.

I would also like to thank the people in the lab who have given me a lot of helps and supports on both scientific and personal matters: Dr. Peter Frick, who initiated this study and has had the most discussions and arguments with me, and whose kindness, insights and expertise I feel indebted to; Mrs. Julie Tai, who has been like a mother to me all these years and whom I can trust anything on; and Dr. Shihka Gaur, who has been always there whenever I need help. I will surely miss the friendly atmosphere of this laboratory wherever I go.

I am grateful to Christopher Thompson and Dr. Suk-Mei Ho for their discussion and kind help on using ProteinChip technology.

My appreciation also goes to my dissertation Committee. Drs. Leonard, Jonassen, Kilpatrick and Stein who are always available for a talk or discussion with me and have spent valuable time on my thesis work.

## ABSTRACT

CIS/SOCS (cytokine-inducible SH2 protein/suppressor of cytokine signaling) are a family of proteins that are thought to act as negative regulators of signaling by erythropoietin, interleukin-6 and other cytokines whose receptors are related to the growth hormone receptor (GHR), and like growth hormone (GH), signal through the JAK/STAT pathway. We examined the possibility that CIS/SOCS proteins may also be involved in GH signaling, in particular, in termination of the transient insulin-like effects of GH. mRNAs for CIS, SOCS3, and to a lesser extent SOCS1 were detectable by Northern blot analysis of rat adipocyte total RNA, and the expression of CIS and SOCS3 was markedly increased 30 min after incubation with 500 ng/ml hGH. Both CIS and SOCS3 were detected in adipocyte extracts by immunoprecipitation and immunoblotting with their corresponding antisera. GH stimulated the tyrosine phosphorylation of a 120 kDa protein (p120) that was co-precipitated from adipocyte extracts along with  $\alpha$ CIS and detected in Western blots with phospho-tyrosine antibodies. However, no tyrosine phosphorylated proteins in these cell extracts were immunoprecipitated with antibodies to CIS3/SOCS3. p120 was later identified as the GHR based on the observations that two GHR antibodies recognized p120 in scale-up experiments and that p120 and the GHR share several characteristics, including their molecular weights, tyrosine phosphorylation upon GH stimulation, interaction with CIS, similar extent of glycosylation as judged by electrophoretic mobility shift after Endo F digestion, comparable mobility shifts upon

thrombin digestion, and N-terminal histidine-tagging. The findings, however, do not rule out the possibility that there might be other tyrosine phosphorylated 120 kDa protein(s) that interact with CIS and contribute to the p120 signal, as well as the GHR.

Further studies of the association of CIS with the GHR revealed that CIS might selectively interact with multiply tyrosine phosphorylated forms of the GHR, and these tyrosines are likely located near the carboxyl end of the GHR. Overexpression of CIS partially inhibited GH-induced STAT5 phosphorylation in CHO cells. Studies in freshly isolated and GH-deprived (sensitive) adipocytes revealed that the abundance of CIS does not correlate with the termination of the insulin-like effects of GH or the emergence of refractoriness. Neither the association of CIS with the GHR nor the tyrosine phosphorylation status of the GHR, JAK2 and STAT5 appear responsible for refractoriness in adipocytes. These data imply that some negative regulators other than CIS might contribute to the termination of GH-induced insulin-like effects in adipocytes.

## TABLE OF CONTENTS

DEDICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
TABLE OF CONTENTS	vii
LIST OF FIGURES	x
LIST OF TABLES	xii
 <b>INTRODUCTION</b>	 <b>1</b>
Synthesis, Secretion and Metabolism of GH	3
Effects of GH on Adipose Tissue	6
Growth Hormone Receptor	8
GHR structure	8
GHR gene	10
GHR isoforms	12
Interaction of GH with the GHR	14
The GHR and the syndrome of GH insensitivity	15
GH/GHR Signaling	16
Receptor dimerization	16
JAK2	18
STAT proteins	20
Other signaling molecules	23
Regulation of GH/GHR Signaling	24
GHR internalization and degradation	24
Phosphatases	25
Cytokine-inducible inhibitors of signaling	25
 <b>SPECIFIC AIMS</b>	 <b>31</b>
 <b>MATERIALS AND METHODS</b>	 <b></b>
Materials	33
Rat adipocytes	34
RNA extraction and Northern Blot analysis	35
Cell culture and transfections	36
Cell stimulation and protein extraction	37
Immunoprecipitation and Western Blotting	38
Immobilization of antibody to protein A agarose	39
Deglycosylation reaction	40
Generation of glutathione S-transferase (GST)-CIS fusion protein and	

preparation of GST beads for pulldown assay	40
Affinity purification of p120 using immobilized GST-CIS	41
Generation of the GHR with a thrombin cleavage site	42
Thrombin digestion reaction	43
Generation of His-tagged GHRs	43
GH binding assay	44

## **RESULTS AND DISCUSSION**

### **Section I Involvement of CIS/SOCS Proteins in GH Actions in Rat Adipocytes**

<b>Results</b>	45
Expression of CIS/SOCS genes in rat adipocytes	45
A tyrosine phosphorylated 120 kDa protein co-precipitates with CIS following GH stimulation in adipocytes	51
CIS complexes with the GHR	54
p120 is a glycoprotein	58
<b>Discussion</b>	59

### **Section II CIS Interacts with the Tyrosine Phosphorylated GHR**

<b>Results</b>	66
Association of p120 and CIS is dependent on GH/GHR	66
Pulldown of p120 by bacterial-produced GST-CIS fusion protein	68
Both p120 and the GHR can be digested by thrombin	70
p120 in cells expressing His-tagged GHR	75
Unsuccessful purification of p120 using GST-CIS pulldown and ProteinChip technology	75
p120 is recognized by GHR antibodies in scale-up experiments	79
Total cellular GHR, tyrosine phosphorylated GHR and CIS-associated GHR	82
<b>Discussion</b>	86

### **Section III Physiological Function Studies of CIS in GH Actions**

<b>Results</b>	91
Identification of the sites on the GHR that interacts with CIS	91
CIS partially inhibits GH-induced STAT5 phosphorylation in CHO cells	94
CIS/GHR association and protein phosphorylation in refractory and sensitive adipocytes	96
Examination of CIS effects on GH/GHR signaling	98
<b>Discussion</b>	102

<b>SUMMARY</b>	113
----------------	-----



<b>FUTURE DIRECTION</b>	115
<b>REFERENCES</b>	117

## LIST OF FIGURES

<b>Figure 1.</b> Illustration of GH actions	2
<b>Figure 2.</b> Schematic representation of the growth hormone receptor	9
<b>Figure 3.</b> Schematic representation of the structures of the hematopoietic cytokine receptor superfamily	11
<b>Figure 4.</b> Illustration of the rat GHR isoforms	13
<b>Figure 5.</b> Illustration of GH signal transduction pathways	17
<b>Figure 6.</b> Illustrated structures of CIS/SOCS proteins	27
<b>Figure 7.</b> Illustration of the hypothesized involvement of CIS/SOCS proteins in GH-induced refractoriness	32
<b>Figure 8.</b> GH up-regulates CIS/SOCS gene expressions in rat adipocytes	46
<b>Figure 9.</b> Detection of basal levels of CIS and SOCS3 proteins in rat adipocytes	48
<b>Figure 10.</b> Regulation of CIS and SOCS3 proteins by GH in rat adipocytes	50
<b>Figure 11.</b> A tyrosine phosphorylated 120 kDa protein (p120) co-precipitates with $\alpha$ CIS following GH treatment in adipocytes	52
<b>Figure 12.</b> Comparison of the immunoblotting sensitivities of Ab2941 and 4G10	55
<b>Figure 13.</b> CIS interacts with the GHR	57
<b>Figure 14.</b> p120 is a glycoprotein	60
<b>Figure 15.</b> GH regulation of Sirp phosphorylation in adipocytes	61
<b>Figure 16.</b> The co-immunoprecipitation of p120 with $\alpha$ CIS is dependent on GHR	67
<b>Figure 17.</b> p120 co-precipitates with bacterial-produced GST-CIS fusion protein	69

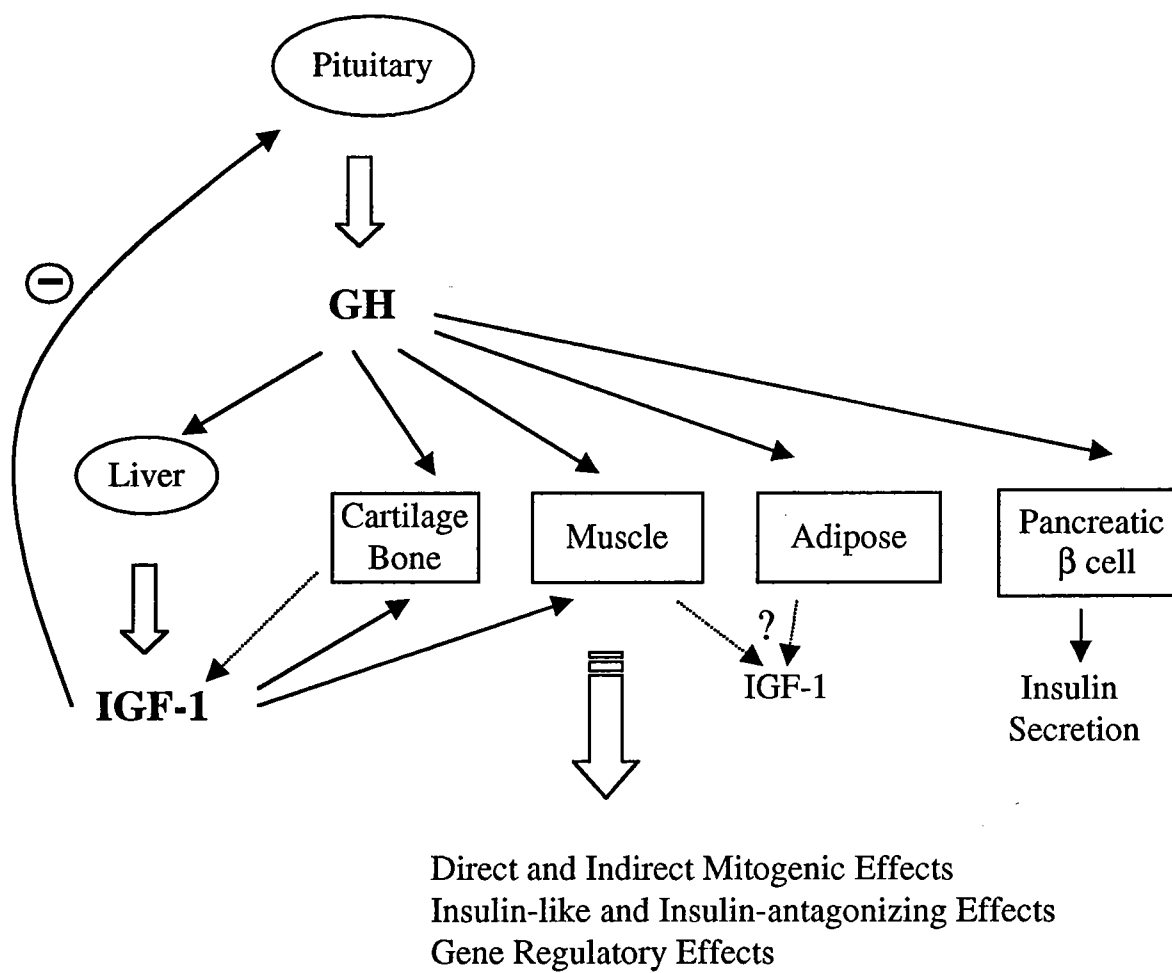
<b>Figure 18.</b> Insertion of a thrombin cleavage site severely affected GH-stimulated GHR phosphorylation	72
<b>Figure 19.</b> Both rat GHR and p120, but not porcine GHR can be digested by thrombin	73
<b>Figure 20.</b> Expression of His-tagged GHRs in 293 cells	76
<b>Figure 21.</b> p120 is recognized by GHR antibodies in scale-up experiments	81
<b>Figure 22.</b> Comparison of tyrosine phosphorylation and protein abundance of CIS-associated GHR and total cellular GHR	84
<b>Figure 23.</b> The tyrosines on the C-terminus of the GHR cytoplasmic domain are required for CIS/GHR interaction	93
<b>Figure 24.</b> Overexpression of CIS in CHO cells partially inhibits GH-activated STAT5 phosphorylation	95
<b>Figure 25.</b> CIS and tyrosine phosphorylations of key GH signaling molecules in adipocytes	97
<b>Figure 26.</b> Effect of CIS on GH signaling	99
<b>Figure 27.</b> Desensitization of phosphorylated proteins stimulated by GH in adipocytes	101

**LIST OF TABLES**

<b>Table 1.</b> The GHR sequences (amino acids 1-50) from different species	74
<b>Table 2.</b> Binding assay of GHR and CIS-associated GHR in adipocytes and 293A cells.	85
<b>Table 3.</b> Effects of CIS on GH-induced STAT5 activity	109

## INTRODUCTION

Growth hormone (GH), also called somatotropin, is a polypeptide hormone secreted by the anterior pituitary gland. GH was discovered and named for its action in promoting normal growth, and was later found to produce a variety of metabolic effects as well. It is the most important hormone for normal growth. In the young, growing individual, GH promotes cell division and differentiation both directly and indirectly through stimulating production of insulin-like growth factor-I (IGF-I). Lack of GH during childhood results in pituitary dwarfism, and overproduction of GH in children results in gigantism, and in adults, acromegaly. GH serves as a metabolic regulator all through life, long after growth has stopped. In this regard, GH accelerates fat mobilization in adipocytes (72) and inhibits glucose utilization in both muscle (7) and adipose tissue (68). GH also dampens insulin-stimulated glucose utilization *in vivo* and may impair glucose tolerance or even induce permanent diabetes mellitus (7). The metabolic effects of GH are exerted largely on skeletal muscle and adipose tissue, which together comprise well over 50% of total body mass and are the major determinants of energy balance. Other targets for related actions of GH are the beta cells of the pancreas, which are stimulated by GH to produce and secrete more insulin, and the liver. The actions of GH at the cellular level include both indirect mitogenic effects exerted through IGF-I (207) and its own direct mitogenic effects in some cells (21), lipogenesis, and anti-lipolytic metabolic effects (70), as well as gene regulatory actions (IGF-I, c-fos, c-jun, Spi2.1, ect.) (119) (45) (80) (203) (Figure 1).



**Figure 1.** Illustration of GH actions.

### **Synthesis, Secretion and Metabolism of GH**

In humans, the 5 to 10 mg of stored GH makes it the most abundant hormone in the pituitary. Ninety percent of the GH produced by somatotropes is composed of 191 amino acids and has a molecular weight of about 22 kDa. The remaining 10%, an alternatively spliced product of the same GH gene, is called 20K GH. The 20K GH has a molecular weight of 20 kDa and lacks residues 32-46. Both forms of hormone are secreted and have similar growth-promoting activity, although the metabolic effects of the 20K form are reduced (159).

About half of the GH in blood circulates bound to the GH binding protein (GHBP), which is identical to the extracellular domain of the growth hormone receptor (GHR, described later). The free or unbound form of GH can readily cross capillary membranes. The GH that crosses the glomerular membrane is reabsorbed and degraded in the kidney. Less than 1% of the hormone secreted each day reaches urine in the recognizable form. Also, GH is degraded in its target cells following uptake by receptor-mediated endocytosis.

According to the somatomedin hypothesis proposed three decades ago, GH action on somatic growth is dependent on an endocrine factor, IGF-I, which is mainly produced by the liver. The existence of IGF-I was first reported by Salmon and Daughaday in 1956 (150). IGF-I was previously referred to as sulfation or thymidine factor, and then somatomedin. Serum IGF-I is tightly bound in a complex that contains two other GH-dependent proteins: the IGF-binding protein 3 and an acid-labile protein. In general, plasma concentrations of IGF-I reflect the availability of GH. They are higher than

normal in blood of persons suffering from acromegaly and are very low in GH-deficient individuals (67). It has been demonstrated and widely accepted that GH's growth stimulating effects are mediated by IGF-I (41). However, the somatomedin hypothesis was challenged by the finding that local administration of human GH *in vivo* to the cartilage growth plate of the proximal tibia of hypophysectomized rats resulted in accelerated longitudinal bone growth (96). This study and later other studies (134)(149)(152)(153) suggest that GH stimulates long bone growth by inducing local production of somatomedin, which in turn stimulates cell proliferation in an autocrine or paracrine fashion (153). It is now well established that GH interacts with several peripheral tissues, including liver, cartilage bone and muscle. Recent studies in the liver-specific IGF-I knockout mouse revealed that though liver-derived IGF-I is the main source of serum IGF-I, it is not required for postnatal growth (201). These studies suggested that autocrine/paracrine-derived IGF-I is more important than liver-derived IGF-I for body growth (109).

In most mammalian species there is a marked sex difference (sexual dimorphism) in the pattern of GH secretion. In male rats, GH is secreted in regular pulses every 3.0 to 3.5 hours with low or undetectable levels between peaks. In female rats the secretion is more frequent, and the baseline levels are higher than in males, resulting in a continuous presence of GH in the circulation that is in contrast to the intermittent presence seen in males. The patterns of GH exposure have dramatic effects on GH-regulated events in the liver (183). For example, the expression of GH-regulated cytochrome P450 enzymes in liver is sexually differentiated (106).



GH secretion is under minute-to-minute control by the nervous system (67). That control is expressed through the hypothalamo-hypophyseal portal circulation, which delivers two hypothalamic neuropeptides to the somatotropes: GH-releasing hormone (GHRH) and somatostatin. GHRH provides the primary drive for GH secretion. Somatostatin reduces or blocks the response of the pituitary to GHRH. Their interaction generates a striking pulsatile pattern of GH release in both humans and experimental animals (124) (177).

In addition to the neuroendocrine mechanisms that control secretion in response to internal or external environment and provide pulsatility under basal conditions, the secretion of GH is under negative feedback control. As with other negative feedback control systems, inhibitory signals are products of GH action, principally IGF-I. Increased concentration of free fatty acids (FFA) or glucose, which are also related to GH action, may also exert inhibitory effects and decrease GH secretion in response to a variety of provocative stimuli. IGF-I appears to act both at the hypothalamic level by stimulating secretion of somatostatin (4) and at the pituitary level by decreasing the response of somatotropes to GHRH (90). Increased FFA and glucose similarly increase somatostatin secretion (95). Some evidence suggests that GH may also have a direct suppressive effect on its own production and may either inhibit the release of GHRH or increase the secretion of somatostatin (139).

### **Effects of GH on adipose tissue**

GH produces a complex array of direct effects on carbohydrate and lipid metabolism in a variety of tissues, among which, skeletal muscles and adipose tissue are the main targets. Under usual physiological circumstances, the metabolic effects of GH are opposite to those of insulin: GH promotes lipolysis in adipocytes and ketogenesis in liver, limits glucose utilization in both muscle and fat, and decreases insulin-sensitivity in muscle, fat and liver. These responses, often classified as diabetogenic, are typically seen only after a lag period of 1-2 hours, and often persist for several hours. Under some circumstances GH may produce opposite effects and act in an insulin-like manner, which has been studied extensively both at the cellular level and in whole animals including humans. These actions include stimulation of glucose metabolism, lipogenesis and anti-lipolysis. They have been called insulin-like because of their similarity to the responses to insulin (71, 78). Insulin-like effects are evident immediately after the addition of GH to adipose tissue obtained from hypophysectomized rats. They are transient and disappear within 1-2 hours and cannot be elicited again for many hours even with high doses of GH. Such insensitivity to insulin-like stimulation by GH is termed refractoriness. In contrast, cells that exhibit GH-induced insulin-like responses (ILR) are referred to as sensitive cells. Characteristically, tissues freshly isolated from normal rats are refractory. However, sensitivity to insulin-like stimulation by GH develops in normal adipose tissue (71) and adipocytes (48) that are maintained in the absence of GH for 3 hours or longer. The ILR to GH can be extended by the presence of inhibitors of RNA or

protein synthesis (69), suggesting that GH may induce some short-lived, regulatory molecules to inhibit ILR.

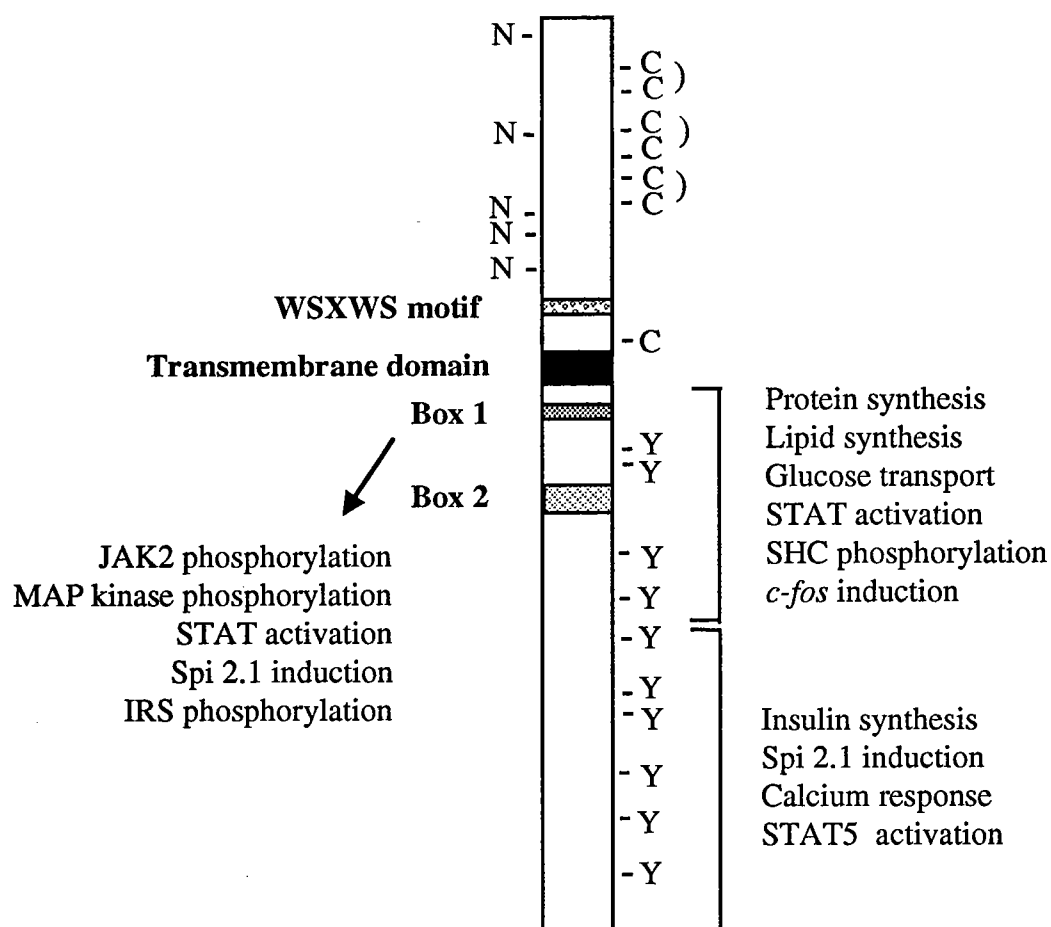
Refractoriness seems to be a separate event from the termination of the ILR. Refractoriness can be produced or prolonged without a preceding ILR by brief exposure to GH at a concentration that is too low to initiate an ILR (70). Conversely, an ILR is not followed by refractoriness if GH is removed by immunoabsorption within 60-90 minutes (70), or if an inhibitor of RNA synthesis is added up to 60 minutes later (73). This suggests that mRNAs and/or proteins that are needed for refractoriness are not synthesized for at least an hour after GH is added. Refractoriness is not due to down-regulation of the GH receptor, since GH binding is undiminished in refractory cells (78).

One of the distinguishing characteristics of refractory and sensitive adipocytes is the difference of the intracellular free calcium level  $[Ca^{2+}]_i$  (155). Cells deprived of GH for just a few hours lower their  $[Ca^{2+}]_i$  by a factor of  $\sim 2$ , presumably as a result of internalization of L-type calcium channels (62). Reintroduction of GH restores the resting calcium level after a lag period of more than one hour by a process that depends upon RNA synthesis and appears to coincide with a shift of calcium channels from internal vesicles to the plasma membrane (62). Adipocytes that are refractory to insulin-like stimulation not only have a higher resting  $[Ca^{2+}]_i$  but also respond to GH with a rapid increase in  $[Ca^{2+}]_i$  resulting from  $Ca^{2+}$  influx through activated L-type  $Ca^{2+}$  channels (63). Interference with the rapid increase in  $[Ca^{2+}]_i$  by  $Ca^{2+}$  channel blockers or chelation of extracellular  $[Ca^{2+}]_i$  with EGTA restores insulin-like sensitivity (156), but the mechanism by which  $[Ca^{2+}]_i$  affects insulin-like sensitivity is not understood.

## Growth Hormone Receptor

For understanding regulation of normal growth and metabolism, it is essential to understand the molecular basis of growth hormone action. At the molecular level, all of the effects of GH are initiated by the binding of GH to its receptor, the growth hormone receptor (GHR). The GHR is a 620-residue single membrane-spanning glycoprotein that binds GH in its extracellular domain and transduces activating signals via its cytoplasmic domain (Figure 2). The GHR was first cloned from rabbit and human liver cDNA libraries (107), and later from liver cDNA libraries of rat (118), mouse (164), cow (86), pig (33), sheep (2) and chicken (23). The GHR is encoded by transcripts of ~4 kilobases (kb) and the GHR gene is comprised of at least 10 exons. The cDNAs encode a ~70kDa protein, which becomes heavily glycosylated and migrates with the mobility of a 114-140kDa protein in SDS-polyacrylamide gels when expressed in mammalian cells. The larger size of the expressed protein cannot be fully explained by glycosylation and may be due to ubiquitination (107).

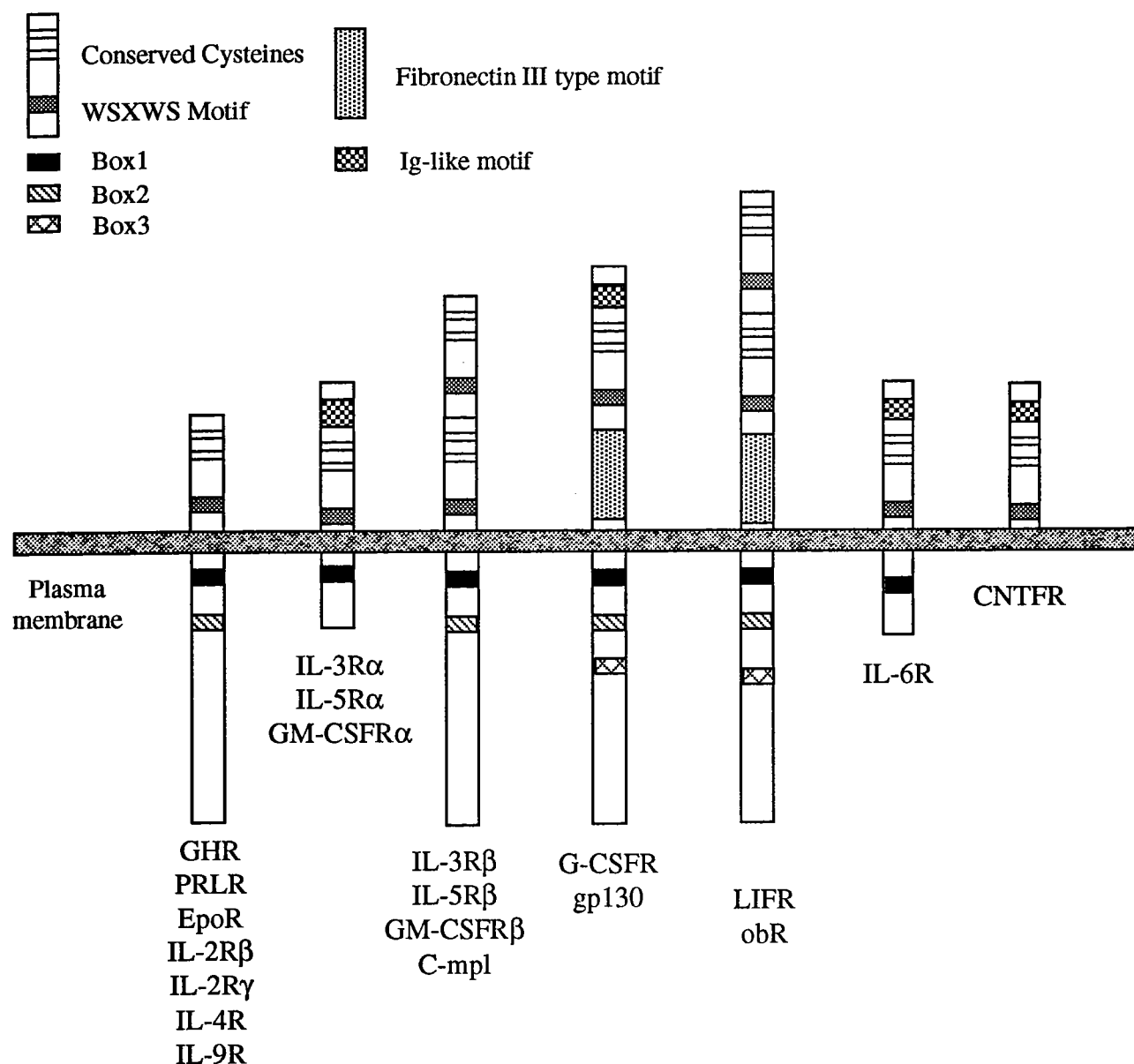
*GHR structure* The GHR belongs to the class I superfamily of cytokine receptors, also called the hematopoietic cytokine receptor family, which includes receptors of prolactin, erythropoietin (Epo) and interleukins (IL) 2-7, 9, 11 and 12, thrombopoietin, leukemia inhibitory factor (LIF), oncostatin, etc. All of these receptors appear to behave similarly with regard to signal generation and transmission and hence clues to the actions of GH have been obtained by comparison with the behavior of other members of the superfamily. Receptors of this family are either single transmembrane proteins or are comprised of one or more subunits which are single transmembrane proteins oriented



**Figure 2.** Schematic representation of the growth hormone receptor. N represents the extracellular asparagine that is a potential N-linked glycosylation site. The 7 extracellular cysteines (C) form 3 cysteine pairs, leaving an unpaired cysteine proximal to the membrane. The sequence of the WSXWS motif in rat GHR is YGEFS. There are 10 cytoplasmic tyrosines (Y) in rat GHR. Regions of GHR shown to be required for various functions are indicated.

with the N-terminus forming the extracellular domain (Figure 3). They are characterized by four conserved cysteine (Cys) residues near the amino terminus and a conserved WSXWS (Trp-Ser-X-Trp-Ser, X can be any amino acid) motif in the carboxyl terminal part of their extracellular domain. The GHR has seven Cys residues, six of which form 3 pairs of intrachain disulfide linkage near the amino terminus. The remaining unpaired Cys is near the transmembrane segment, and although it is a highly conserved feature of the cytokine receptor superfamily, its function has not been elucidated. The WSXWS motif in GHR contains conservative substitutions (YGEFS) and has been postulated to play a critical role in ligand binding by providing structural stability (43), though it does not contact the ligand. The cytosolic domain contains no consensus enzymatic domain, and hence no catalytic activity was found. However, two motifs, Box 1 and Box 2, are relatively conserved in the cytoplasmic membrane-proximal domain for most receptors of this superfamily. Box 1 is proline-rich and consists of eight amino acids ( $\Psi$ -X-X-X-Al-P-X-P, where  $\Psi$  represents hydrophobic residues and Al represents aliphatic residues). In the mammalian GHR, Box 1 contains ILPPVPVP (27). Box 1 is the site of Janus Kinase 2 (JAK2) binding for the GHR (56) and is essential for most of GHR's signaling function (see JAK2). Box 2 is less well conserved and begins with a cluster of hydrophobic amino acids, and ends with one or two positively charged amino acids (127). Deletion or mutation of Box 1 and Box 2 abrogates proliferative signaling in members of this superfamily (92).

*GHR gene* The GHR gene is located on chromosome 5, p13.1/p12, in humans (14), adjacent to the *myc* oncogene on chromosome 15 in the mouse (49), and on

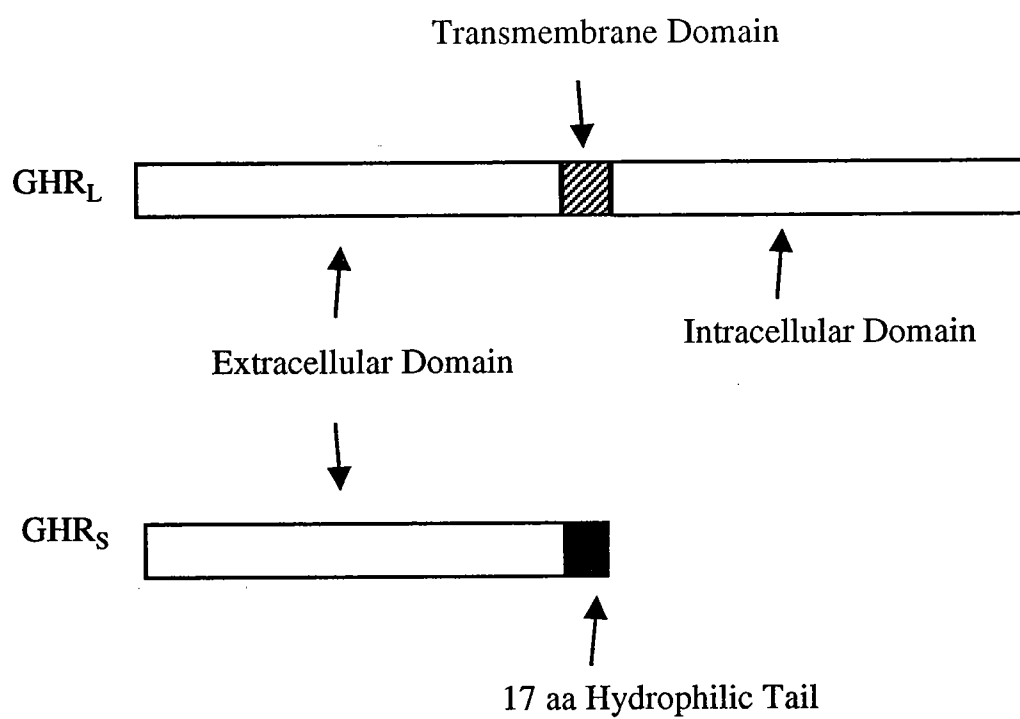


**Figure 3.** Schematic representation of the structures of the hematopoietic cytokine receptor superfamily. PRLR: prolactin receptor; EpoR: erythropoietin receptor; IL-2R, ...IL-9R: interleukin 2...9 receptor; GM-CSFR granulocyte-macrophage colony stimulating factor receptor; c-mpl: c-myeloproliferative leukemia virus or orphan receptor of unknown ligand; G-CSFR: granulocyte colony-stimulating factor receptor; gp130:  $\beta$  subunit of IL-6R; LIFR: leukocyte inhibitory factor receptor; obR: obese receptor; CNTFR: ciliary neurotrophic factor receptor.

chromosome 16 in the pig (32). The prolactin receptor gene is located in the same region as the GHR gene in humans (11). The GHR gene comprises more than ten exons, extending over at least 87 kb, with the receptor itself being encoded by nine exons (66). Exons 2-9 range in size from 66 to 179 base pairs (bp), whereas exon 10 which encodes nearly all of the cytoplasmic domain and a long 3' untranslated region (UTR), is about 3400 bp. Exon 2 encodes the signal peptide and the N-terminal six amino acid residues. Exons 3-7 encode the rest of the extracellular domain. Exon 8 encodes the transmembrane domain and the membrane proximal four residues of the cytoplasmic domain. Exon 9 includes coding for Box 1 but not Box 2. Multiple tissue specific sequences have been identified in exon 1, which encodes the 5'UTR. Existence of several splice variants has been reported (137), as well as a liver-specific promoter (exon 1A) (16) (168) (214) and exon 1B(1).

*GHR isoforms* Two principal products of the GHR gene are formed in most if not all mammalian species and arise either as a result of alternative splicing of mRNA or post-translational processing. In addition to the full length GHR, a soluble isoform of GHR known as the GH binding protein (GHBP) circulates in the blood. The soluble GHR is generated differently among species. In rodents, alternative splicing gives rise to a 1.2 kb mRNA encoding the short form GHR ( $GHR_s$ ), in which the transmembrane and cytoplasmic domains of the full length receptor ( $GHR_L$ ) are replaced by a unique 27-hydrophilic residue carboxyl tail in mouse (164) or a 17-residue in rat (17) (57) encoded by Exon 8a (210) (Figure 4). Non-rodent species apparently lack the alternate exon or alternative splicing sites, but produce a circulating binding protein by proteolysis of





**Figure 4.** Illustration of the rat GHR isoforms.

either the full length GHR (165) or a truncated isoform arising from alternative splicing in the cytoplasmic domain of the GHR (40). Although some GHR<sub>s</sub> circulates in rodent blood as the GHBP, some is also retained in tissues, where the amount of GHR<sub>s</sub> recovered from cell membrane fractions exceeds that of GHR<sub>L</sub> (57). The GHR<sub>s</sub> retained in tissues appears to be incompletely glycosylated and lacks the terminal sialic acid found in GHR or GHBP (58). In rat adipocytes and cultured 3T3-L1 cells GHR<sub>s</sub> contributes to surface binding of GH (59). The manner by which GHR<sub>s</sub> is tethered to membranes is unknown, and it is possible that GHR<sub>s</sub> might have a signaling function. GHR<sub>s</sub> may help mediate signal transmission initiated by ligand binding to GHR<sub>L</sub>, or enhance or diminish the GHR<sub>L</sub> mediated GH effects. A short form of GHR was also found in tissues of non-rodent species. It is noteworthy that other members in the cytokine receptor superfamily also have several isoforms (147), such as prolactin receptor, leptin receptor and receptors of IL-2 and IL-6, and that some of these are soluble and circulate in blood.

*Interaction of GH with the GHR* The interaction of GH with GHR though complicated, is now understood structurally with a high degree of resolution, owing greatly to a brilliant series of crystallographic and kinetic studies employing recombinant GH and the extracellular domain of the GHR. These studies have elegantly demonstrated that GH interacts with the GHR to form a complex of 1:2 GH:GHR stoichiometry (38, 43). Although GH is a molecule composed of four antiparallel helical bundles without an axis of symmetry, two distinct sites within a single molecule of the hormone engage two GHR at nearly identical contact points in the extracellular domain on each receptor (43). GH binds sequentially to one receptor molecule at the so-called site 1 on GH and then to

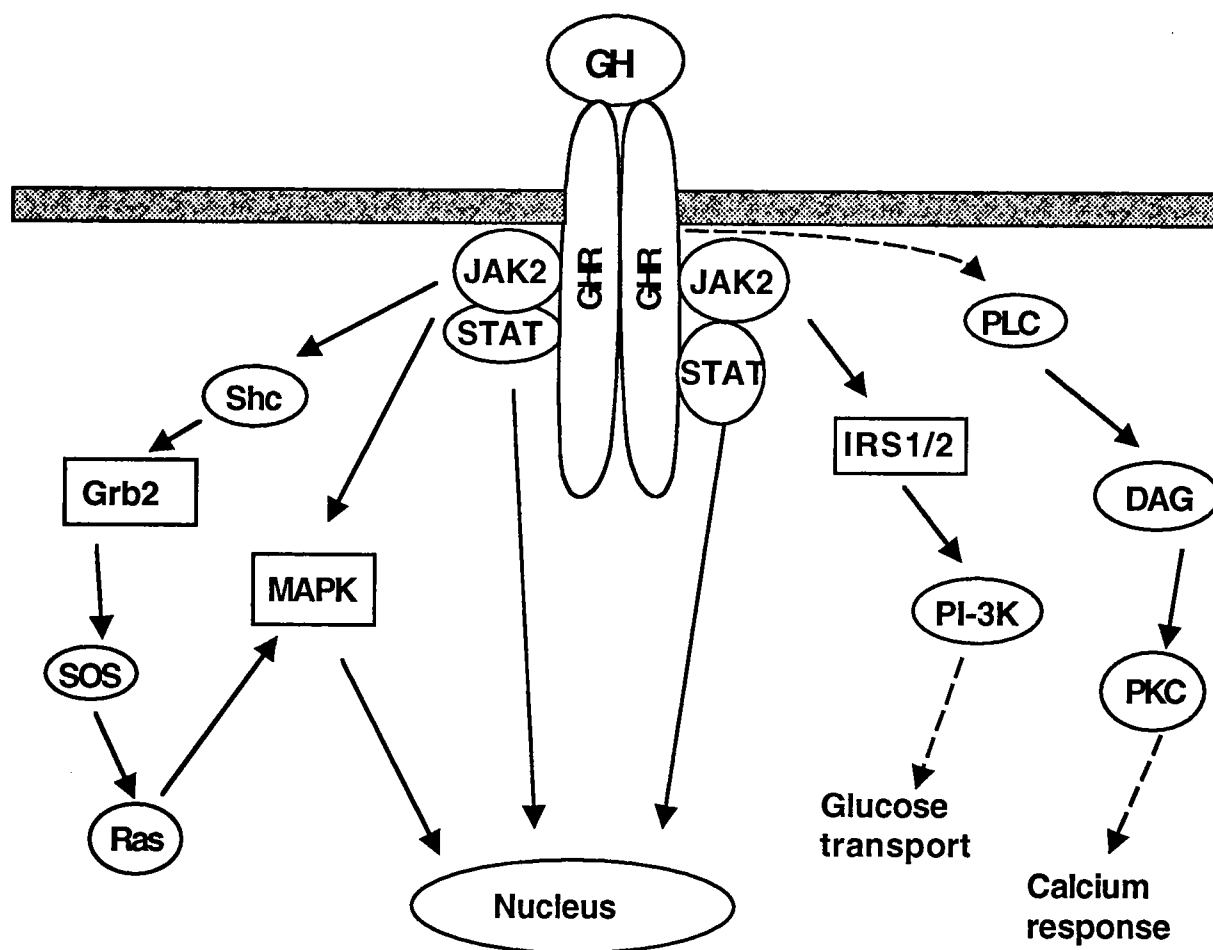
a second receptor at site 2 on GH (60), which produces a receptor dimer that initiates signaling. Binding is ordered because of a stronger interaction between GHR and site 1 than site 2 of GH. GH:GHR<sub>2</sub> complex is further stabilized by direct interaction of the GHRs between the GH binding sites and the transmembrane regions (43). This region is sometimes called the dimerization domain.

*The GHR and the syndrome of GH insensitivity* Growth hormone insensitivity is an autosomal recessive disorder with elevated GH levels in blood associated with a reduction or abolition in the biological actions of GH (151). Laron dwarfism (105) is a primary representative of GH insensitivity in human. Patients with this syndrome have a clinical phenotype of severe growth retardation with high circulating levels of GH accompanied by low serum IGF-I and IGF binding protein 3, with no responsiveness to exogenous GH. It is caused by mutations in the GHR gene, mainly in the extracellular domain. The majority of GHR mutations resulting in GH insensitivity are thought to affect GH binding, hence the finding of low levels of GHBP in many patients. Interestingly, homozygous GHR/BP knockout mice showed severe postnatal growth retardation, proportionate dwarfism, greatly decreased serum IGF-I and elevated serum GH concentrations, which represent the characteristics of the phenotype typical of individuals with Laron syndrome (211) and also individuals with congenital absence of GH. This further confirmed the primary role of the GHR in GH's growth promoting effects.

## GH/GHR Signaling

Our knowledge of GH/GHR signal transduction has been greatly expanded in the past decade of research following the cloning of the GHR and the discovery of tyrosine phosphorylation as a mechanism for signal transduction. More and more protein molecules have been discovered to be involved in GH signaling (Figure 5). These proteins may participate in signal transduction through several pathways and these pathways may intersect with each other and with signal transduction pathways associated with other ligands.

*Receptor dimerization* As mentioned above, one GH molecule complexes with two molecules of GHR. The binding of GH to GHR appears to be sequential in the kinetic studies described by Cunningham et al (38), leading to the hypothesis that GH binding causes GHR dimerization. Mutated GH (G120R) in which the highly charged R (arginine) disrupts the second helix, and hence site 2, fails to induce GHR dimerization. G120R is not only biologically inactive, but is also an antagonist of GH activity (29). This finding is consistent with the idea that GH-induced dimerization of GHR is required for GH action (34) (175) (85). Another important observation supporting GHR dimerization comes from the bell-shaped dose-response curve for GH-induced proliferation seen with increasing concentration of GH. Receptor dimerization would predict that at high hormone concentrations, receptors bound at site 1 interactions with GH would be unavailable to bind at site 2 thus preventing formation of effective signaling dimers (93). Two GHRs can also form a disulfide linkage through their unpaired cysteines. In human IM-9 lymphocytes expressing GHR, formation of GH-



**Figure 5.** Illustration of GH signal transduction pathways. PLC: phospholipase C; DAG: diacylglycerol; PKC: protein kinase C; IRS1/2: insulin receptor substrates 1 and 2; PI-3K: phosphatidylinositol-3 kinase; MAPK: mitogen-activated protein kinase; SOS: son of sevenless.

dependent disulfide linkage between GHR was found to be rapid and quantitatively significant (56). However, GH-induced GHR disulfide linkage does not appear to be required for GHR dimerization (209).

Dimerization of the GHR upon ligand binding is typical of the behavior of the cytokine receptor superfamily and provides a basis for understanding how hormone binding can be translated into signal generation by cytokine receptors, as well (198). It has been shown that JAK2 activation and GH-enhanced association of JAK2 with GHR depend more on GHR dimerization than on tyrosine phosphorylation of GHR and/or JAK2 (209). The question of whether or not receptor dimerization is sufficient for GH signal transduction has not been settled. Crystal structure studies revealed that the ligand-receptor complex undergoes conformational changes following receptor dimerization (34). The conformational changes resulting from the binding of GH to two GHR may be important for GH signaling, perhaps by increasing the affinity for JAK2 or other associated proteins.

**JAK2** Over the past several years, a variety of cytoplasmic tyrosine protein kinases have been implicated in cytokine signaling. Of these, the most critical kinases apparently are the *Janus* family of protein kinases (JAKs) (91). The JAKs were initially identified through approaches to discover novel tyrosine kinases and therefore, their role in cytokine signaling was not immediately appreciated. To date, four mammalian members of the JAK family have been identified, consisting of ubiquitously expressed JAK1, JAK2 and TYK2, and JAK3, which is primarily expressed in hematopoietic cells. Activation of JAKs appears to be a common signaling event that occurs in response to

ligand binding to members of the cytokine receptor superfamily. However, different cytokines activate different combinations of JAKs, providing one mechanism by which specificity in response to multiple cytokines may be accomplished. Even though the same JAK is activated by multiple cytokines, the level of activation varies substantially in response to stimulation by different cytokines, as does the time course of the response (161). Initially, JAK2 was thought to be the only kinase engaged in GH signaling. Later studies showed that JAK1 and TYK2 may also be activated (162) (87), to a lesser extent, and thereby may mediate some of the effects of GH.

The activated JAK2 has been shown to phosphorylate several intracellular substrates including GHR, JAK2 itself, as well as transcription factors of the STAT (signal transducer and activator of transcription) family (28) (123) (186). Studies using truncated and mutated GHR have implicated the Box 1 motif as an indispensable component for GH-dependent association of JAK2 with GHR and for tyrosine phosphorylation and activation of JAK2 (56) (167) (187) (193). More distal regions of the GHR appear to augment the interaction (56) (167) (178). The mechanism by which GH activates JAK2 is unknown. Forced receptor homodimerization of only the transmembrane and intracellular domains of the GHR by insertion of a leucine zipper can lead to constitutive activation of known end points of GH signaling in engineered cells, supporting the view that proximity of JAK2 to the GHR is the essential element in GH signaling (19). It is not clear if JAK2 is constitutively bound to the GHR, or is recruited to the GHR upon the binding of the ligand to the receptor, nor is it known if the activation mechanism for JAK2 is due to a conformational change, or tyrosine

phosphorylation or both. One hypothesis consistent with what is known about GHR structure and tyrosine kinase activation in general is that binding of GH to two GHR molecules increases the affinity of JAK2 for each GHR and allows two JAK2 molecules to come into sufficiently close proximity to transphosphorylate one or more tyrosines in the kinase domain of the paired JAK2, thereby activating JAK2 (27). JAK2 activation appears to be the general initial signaling event for all GH responses described so far except for the increase in intracellular  $[Ca^{2+}]$  in Chinese hamster ovary (CHO) cells (20).

*STAT proteins* Initially identified in the interferon signaling pathway (39), STAT proteins are latent cytoplasmic proteins containing Src homology (SH) 2 and SH3 domains. They participate in cytokine signaling by regulating the expression of early response genes. The current model of STAT activation (91) consists of the initial recruitment to the receptor complex through interaction of the SH2 domain of the STATs with phosphorylated tyrosines on the receptor. This interaction provides the specificity with which the cytokine activates specific STAT(s). The next step involves the tyrosine phosphorylation of the STAT by the associated JAK. Once phosphorylated, the STAT protein forms homodimers or heterodimers with other STAT proteins, translocates to the nucleus, binds to DNA, and activates or represses transcription of target genes (113).

Seven mammalian STAT proteins have been identified, many of which play highly specific roles in innate and acquired immunity. STAT1 is critical for IFN-induced viral resistance (47, 122). Similarly, STAT6 specifically mediates the effects of IL-4 or IL-13 on B or T cells (98, 158), while STAT4 is critical for IL-12 signaling (99) (180). STAT3, when deleted, results in a very early embryonic lethality due to unknown



deficiencies. STAT5 was initially identified as a prolactin-induced mammary gland transcription factor (190). Two STAT5 genes encode proteins that are approximately 95% identical in amino acid sequence: STAT5a and STAT5b (110). The two proteins differ primarily in their C-terminal transcription activation domains. The differences are also exhibited in their DNA binding specificities and tissue distributions.

Association of STATs with GHR has been detected in 3T3-F442A cells and mouse L cells (200). Binding of STAT with the GHR presumably positions it optimally for its interaction with JAK2. GH has been shown to activate STAT1, 3, 5a and 5b in various tissues and types of cultured cells (reviewed in (88)). In the *c-fos* promoter, GH induces the binding of three complexes to the Sis-inducible element (SIE) (25) (79) (123) which contains binding sites for STAT1 and STAT3 homodimers and STAT1/3 heterodimers. GH also stimulates STAT5 binding to the IFN $\gamma$ -activated sequence (GAS) in the Spi2.1 gene (10). GH-dependent activation of STATs requires JAK2 activation. However, though JAK2 activation appears to be sufficient for STAT phosphorylation and DNA binding when JAK2 and STATs are overexpressed (166) (202) (162) or in a cell-free setting (76), it does not appear to be sufficient for STAT activation in intact cells. Current evidence suggests that STAT5 activation by GH requires phosphorylation of specific tyrosine residues within the GHR. Residues Tyr487, Y534, Tyr566 and Tyr627 in porcine GHR are required for GH-dependent tyrosine phosphorylation of STAT5 (194) (84). In addition, the residue Tyr333 and/or Tyr338 in the juxtamembrane area of the rat GHR may play a role in GH-dependent activation of STAT5a and 5b (162) (163).

In addition to tyrosine phosphorylation, recent studies show that serine phosphorylation also plays a role in STAT activation or regulation (140). MAP kinase has been shown to phosphorylate STAT5a and this phosphorylation is required for full activation of GH-induced STAT5a (138). STAT1, 3, 5a and 5b contain multiple consensus sites for phosphorylation by protein kinase C and casein kinase. Thus, it is possible that multiple kinases could activate STAT proteins and contribute to the regulation of GH signaling.

The physiological importance of STAT 5a and 5b was further determined by studies in knockout mice. STAT5a appears to be required for mammary gland development and lactogenesis, two processes mediated by prolactin. No effect on body growth was noted in STAT5a-deficient mice (111) (179). In comparison, STAT5b-deficient mice have pronounced impairment in body growth, especially in males. Serum IGF-I levels were reduced in males but not in females. STAT5b gene disruption also leads to a major loss of multiple sexually differentiated responses associated with the sexually dimorphic pattern of pituitary GH secretion. Male-specific gene expression in the liver is decreased to wild-type female levels in STAT5b-deficient males, while female-predominant liver gene products are increased in males to near female levels (179). Thus, STAT5b was proposed as a key intracellular mediator of the stimulatory effects of GH pulses on male-specific liver gene transcription (197). STAT5b was also reported to be involved in the lipolytic action, but not in the insulin-like effects, of GH on adipose tissue (54). STAT5a/b double knockout mice were also growth retarded, and the phenotypes are quite similar to those observed in GH-deficient (46) or GHR-deficient

(211) mice. These studies demonstrate that the two STAT5 proteins have an essential, and often redundant, role in a spectrum of physiological responses associated with growth hormone and prolactin (179). Thus, both STAT5 proteins, acting in concert, are required for normal GH-dependent growth.

*Other signaling molecules* In addition to JAK and STAT proteins, several other signaling molecules appear to be involved in GH signal transduction (Figure 5). The MAP kinases ERKs 1 and 2 have been shown to be tyrosine phosphorylated after GH treatment (26) (199) (12). GH-induced phosphorylation of Shc proteins is thought to lead to the activation of Ras-MAP kinase pathway (185). GH also stimulates the tyrosine phosphorylation of the insulin receptor substrates IRS1 and IRS2 and their subsequent binding to the 85 kDa regulatory subunit of phosphoinositol 3 (PI-3) kinase (143, 169). These reactions probably initiate the insulin-like responses to GH. However, no difference of phosphorylation of IRS1 was observed between refractory and sensitive adipocytes, suggesting therefore, that induction of refractoriness may be distal to IRS1 phosphorylation or perhaps that the specific tyrosines phosphorylated may be different in sensitive and refractory cells (169). The PI-3 kinase inhibitor wortmannin blocks the ability of GH to stimulate lipid synthesis in rat adipocytes (144). Phospholipases that lead to formation of diacylglycerol and activation of protein kinase C are also regulated by GH (146) (160). GH also causes an increase in  $[Ca^{2+}]_i$  in freshly isolated adipocytes (155) (156), IM-9 lymphocytes (94), insulin secreting INS-1 cells (157), rat hepatocytes (116) and in CHO cells expressing rat GHR (20) (84). Mutagenesis studies suggest that calcium signaling may be independent of JAK2 activation, since Box1 is not required for

the GH-dependent calcium increase in CHO cells (20). Recently, it has been shown that a variety of proteins that are involved in the regulation of the cytoskeleton are also regulated by GH. These proteins include focal adhesion kinase (FAK), paxillin, tensin, CrkII, c-Src, c-Fyn, c-cbl and Nck (213) (212).

### **Regulation of GH/GHR Signaling**

The actions of cytokines are limited in both duration and magnitude, making it important to understand the mechanism by which their actions are negatively controlled. However, compared to the understanding of the stimulation of JAK-STAT pathway by cytokines (including GH), our knowledge of the termination of these signals is very limited. The high affinity and rapid irreversible binding of GH to its receptor results in reduction in available receptor concentration, which in turn would decrease the response of cells to the ligand stimulation. In addition to the rapidity of receptor turnover, in the current view, there are at least 3 other aspects that could contribute to the termination of GH/GHR signaling: GHR internalization and degradation, dephosphorylation of phosphorylated tyrosines on proteins such as GHR, JAK2 and STATs by phosphatases, and expression of negative regulators such as CIS/SOCS proteins (see below).

*GH internalization and degradation:* In the absence of ligand, GHR has a short half-life of 45 minutes to 2 hours (74) (148) (18) (128) depending on the cell system used (45 minutes in rat adipocytes (74)). In the presence of ligand, the dimerized receptors are found to be ubiquitinated, endocytosed and degraded (173), and such endocytosis and degradation of GHR is proteasome-dependent (184). It was also reported that an intact ubiquitination system was required for activation of the GH-induced JAK-STAT

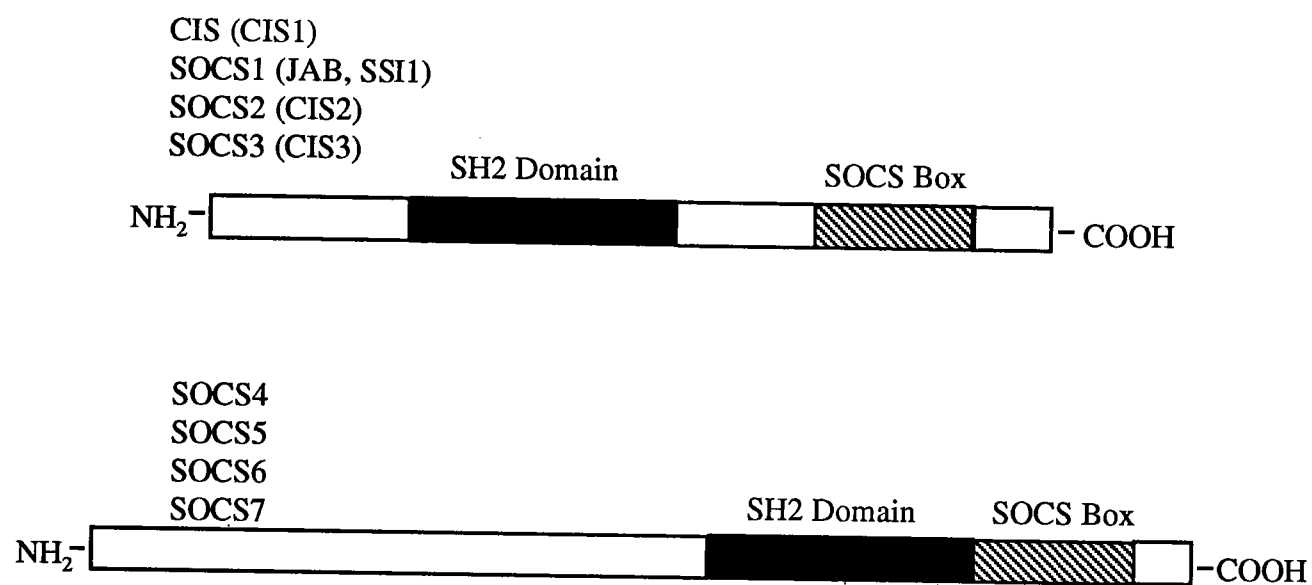
signaling pathway (174). In rat adipocytes, the endosomal/lysosomal pathway is also operative for GHR degradation, since inhibitors of lysosomal acidification increase the half-life of  $^{125}\text{I}$ -hGH bound to receptors (75).

*Phosphatases:* Because tyrosine phosphorylation plays such an important role in cytokine signaling, phosphatases are among the first candidates that have been examined for their ability to down-regulate cytokine signaling. The SH2-containing phosphotyrosine phosphatase SHP-1 has clearly been demonstrated to down regulate Epo signal transduction (103). SHP-1 directly associates with JAK2 (97) and has been suggested to play a role in the dephosphorylation of JAK2/STAT in liver in response to GH (81). Experiments with the related phosphatase, SHP-2, however, showed that it associates with GHR and JAK2, and implied a positive role of SHP-2 in GH signaling (101), perhaps by acting as an adaptor protein in the signal complex. Both SHP-1 and SHP-2 have been suggested as potential phosphatases that deactivate STAT5 before (206) or after STAT5 translocates to nucleus (141). GH stimulates the association of SHP-2 with Sirp (signal-regulatory protein) (172), which appears to negatively regulate GHR/JAK2 signaling (171). Other unidentified phosphatases were also suggested to be involved in dephosphorylation of GH/GHR signaling molecules (65).

*Cytokine-inducible inhibitors of signaling* CIS proteins (cytokine-inducible SH2 protein) are a family of cytokine-inducible inhibitors of signaling. They have also been called suppressor of cytokine signaling (SOCS) or STAT-induced STAT inhibitor (SSI). Currently, there are 8 known members in this family: SOCS1 (also called JAB for JAK binding protein, or SSI-1), CIS (CIS1), SOCS (CIS) 2, 3, 4, 5, 6 (117) and 7 (132). All

members have a conserved 40-amino-acid SOCS box (approximately 50% identity) near their carboxyl termini and an SH2 domain amino-terminal of the SOCS box (Figure 6). The function of SOCS box remains unknown. In SOCS1/JAB, the SOCS box is not required for its inhibitory activity but rather seems to be involved in protein stability (130), possibly by interacting with elongins B and C which may target proteins to destruction by the proteosome (208). The N-termini exhibit little sequence identity among CIS/SOCS proteins. It was found that both the N-terminus and the SH2 domain of SOCS-1 were required for suppression of IL-6 and LIF signaling and inhibition of JAK activity (133). Recently, it was shown that the N-terminal domain but not the SOCS box confers specificity in terms of suppressing a GH-induced STAT5 responsive reporter gene construct (83).

Studies of CIS/SOCS proteins revealed a number of differences in their expression patterns. SOCS1 is mainly expressed in thymus, spleen and lung whereas CIS is ubiquitously expressed (170). CIS has a particularly high expression level in fat tissue, and also in kidney and muscle (182). Second, CIS can be induced by a wider range of cytokines than those that induce the other family members. Originally reported to be induced by Epo, IL-2 and IL-3, CIS has also been shown to be induced by IL-4, IL-7, IL-13, thrombopoietin (Tpo), granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN- $\gamma$ , TNF- $\alpha$ , IL-1, macrophage colony-stimulating factor (M-CSF), IL-6, IL-12, LIF (170) and GH (3). Only a small subset of these stimuli induce SOCS1. SOCS2 and SOCS3 are somewhat more broadly expressed than SOCS1, with SOCS3 showing a pattern of induction similar to that of CIS (170).



**Figure 6.** Illustrated structures of CIS/SOCS proteins.

Experiments with CIS/SOCS proteins suggest that they function to negatively regulate signal transduction. Induction of expression of the CIS/SOCS proteins has been reported to occur in response to various cytokines, and once induced, each member of the CIS/SOCS family proteins appears to inhibit signaling in a different way. There does not seem to be a universal target for these proteins. The first member of this family, CIS, was originally identified as an immediate early response gene induced by IL-3 and Epo in Ba/F3 cells (205). The CIS gene is a direct target of STAT5 (120), and its product binds to the tyrosine phosphorylated IL3 receptor and Epo receptor (205). It partially suppresses STAT5 activation in HEK 293 cells and Ba/F3 cells. It was suspected that CIS could suppress STAT5 phosphorylation by competing for and masking tyrosine phosphate docking sites for STAT5 on the receptor (120). However, no competition for binding to the GHR between STAT5 and CIS proteins was observed (83). Consistent with a potentially important physiological role of CIS in GH signaling, transgenic mice constitutively overexpressing CIS exhibited growth retardation, less adipose tissue, inhibition of GH-mediated gene expression in liver, as well as defects in the IL-2 response of T cells (121), which are similarly observed in mice deficient of STAT5 (179).

SOCS1 was identified as a factor capable of inhibiting IL-6-induced differentiation of monocytic leukemic M1 cells into macrophages (170). At the same time JAB was cloned based on its ability to interact with the kinase domain of JAK2, and SSI-1 was cloned based on homology to the SH2 domain of STAT3 (129). Analysis of the sequences of SOCS1, JAB and SSI-1 revealed that these proteins were identical. Later studies demonstrated that SOCS1 inhibits all four JAK family members when



overexpressed in COS or HEK 293 cells (52). SOCS1 also inhibited IL-6 induced tyrosine phosphorylation of STAT3, the IL-6 signaling subunit gp130, and JAK2 (176). However, CIS and SOCS3 were unable to inhibit the intrinsic kinase activity of JAK2. Therefore it was proposed that they inhibit cytokine signaling at a step distal to JAK activation. Later it was found that the inhibition of SOCS3 on JAK2 activity is GHR-dependent (83) (142). Deletion of JAB/SOCS1 in mice results in perinatal lethality that is related to its inhibition to interferon  $\gamma$  activation (5, 115), whereas deficiency of SOCS3/CIS3 in mice causes embryonic lethality associated with marked erythrocytosis (114). SOCS2 was found to interact with the insulin-like growth factor-I (IGF-I) receptor in mouse fibroblasts and HEK 293 cells (44). Human SOCS3, which has a 90% nucleotide and 97% amino acid homology to murine CIS, inhibits STAT3 phosphorylation and cell differentiation in LIF-treated M1 cells. Overexpression of SOCS3 in corticotroph AtT-20 cells inhibits LIF-induced phosphorylation of gp130 (signaling subunit of the LIF receptor) and STAT3, ACTH secretion, and POMC gene expression (13). SOCS3 gene expression was found to be preferentially induced by GH in mouse liver and 3T3-F442A cells. In addition, expression of SOCS1 and SOCS3, but not CIS and SOCS2, inhibited the ability of GH to regulate GH-responsive gene expression in CHO cells (3). Furthermore, SOCS3 was recruited to gp130 at the SHP-2 binding site directly or through SHP-2, and the activities of the two inhibitors, SOCS3 and SHP-2, appeared to be functionally linked (154) (131). Recently it was reported that insulin, whose receptor does not belong to the cytokine receptor superfamily, induces the expression of SOCS3 gene as well. SOCS3 inhibits insulin activation of STAT5b without

modifying the insulin receptor kinase activity, possibly by competing for the STAT5b binding motif in the receptor (50)

In addition to inhibiting activities of kinases or other phosphorylated signal molecules of cytokines, CIS/SOCS proteins apparently can modulate signaling by another mechanism: stimulating protein degradation. The 37 kDa form of CIS is ubiquitinated and might be involved in directing the EpoR/CIS complex to the proteasome-mediated degradation of the EpoR (188). SOCS1 was also found to target the hematopoietic specific guanine nucleotide exchange factor, VAV, for ubiquitin-mediated protein degradation (42).

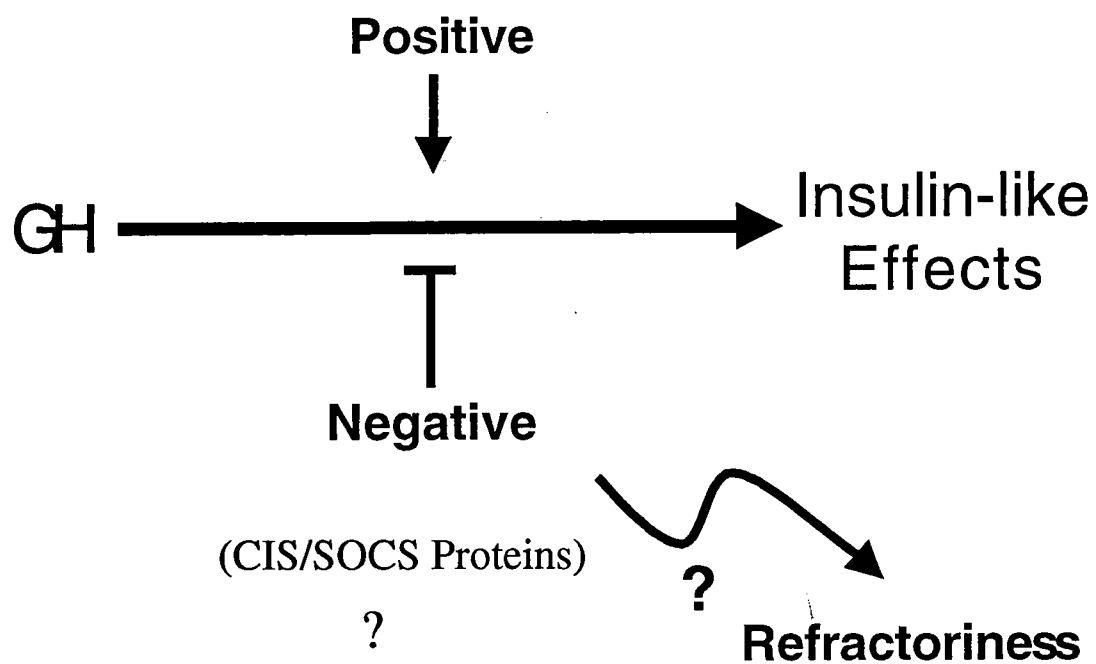
The data suggest that CIS/SOCS may act through multiple mechanisms to regulate cytokine signaling. Elucidation of the mechanisms and determination of their physiological importance will greatly enrich our understanding of signal transduction pathways of cytokines in general, and of GH, in particular.

## **SPECIFIC AIMS**

As described earlier, adipose tissue is a major target of GH action. Compared to other GH target tissues, its lack of receptors for IGF-I facilitates the study of the direct actions of GH without the complication of the autocrine effects of IGF-I. Refractoriness refers to insensitivity of adipose tissue or adipocytes to insulin-like stimulation by GH. The mechanism for refractoriness is unclear. It is possible some molecules can negatively regulate certain GH actions and thereby suppress the insulin-like effects of GH. Discovery of molecules responsible for such suppression will not only clarify the mechanism leading to refractoriness, but also help to better understand the biological functions of GH. The function of CIS/SOCS proteins as negative regulators of cytokine signaling makes them good candidates for proteins that might regulate insulin-like sensitivity and refractoriness of adipocytes (Figure 7).

This study was initiated to investigate the functions of CIS/SOCS proteins in GH actions, in particular, in refractory phenomenon. The specific aims of this study are:

- I. To determine if GH regulates CIS/SOCS protein gene expression in rat adipocytes.
- II. To identify molecules that CIS/SOCS proteins interact with upon GH stimulation.
- III. To investigate if CIS/SOCS proteins are involved in GH actions and in refractoriness.



**Figure 7.** Illustration of the hypothesized involvement of CIS/SOCS proteins in GH-induced refractoriness.

## MATERIALS AND METHODS

### *Materials*

The plasmids pcDNA3/*myc*-CIS/SOCS that contain the cDNA for CIS proteins (117) and the polyclonal antibodies for CIS and SOCS3 (205) were kindly provided by Dr. A. Yoshimura from Kurume University, Kurume, Japan. Plasmids pLM108rGHR and pcDNAIamp-rGHR which contain full length cDNA of rat GHR (rGHR) were kindly provided by Dr. N. Billestrup (Hagedorn Research Institute, Gentofte, Denmark). Plasmids pMet/pGHR, TR4 and Fc8 which contain wild type, mutated or truncated porcine GHR cDNA were provided by Dr. X. Wang from Massachusetts General Hospital, Boston, MA. Plasmid pGEX 4T-3 was purchased from Amersham/Pharmacia Biotech (Piscataway, NJ). All oligonucleotide primers were synthesized by Gibco Life Technologies (Rockville, MD).

Rabbit polyclonal anti-GHR Ab2941 that was raised against the intracellular domain of the rat GHR fused to the maltose binding protein was prepared in this laboratory (57) and used for Western blotting analysis. The affinity purified Ab2941 was used for immunoprecipitation. Rabbit polyclonal anti-GHR BB74 was kindly provided by Dr. W. R. Baumbach (American Cyanamid, Princeton, NJ). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine monoclonal antibodies: 4G10 was from Upstate Biotechnology (Lake Placid, NY), PY99 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HRP conjugated anti-*myc* polyclonal antibody was purchased from Invitrogen (San Diego, CA). Spent medium of anti-*myc* hybridoma 1-9E10 culture was

kindly provided by Dr. J. Leonard (University of Massachusetts Medical Center, Worcester, MA). Anti-Sirp antiserum was a gift from Dr. S. Frank (University of Alabama at Birmingham, Birmingham, AL). Anti-rabbit Ig, HRP-linked whole antibody was purchased from Amersham/Pharmacia Biotech (Piscataway, NJ) and used as secondary antibody in Western blot analysis. All other polyclonal antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

293A cells are human embryonic kidney (HEK) 293 cells that stably express high levels of the rat GHR. They were prepared by Dr. G.P. Frick in this laboratory. Parental CHOK1, CHOA (stably express the 120 kDa rat GHR) and CHO4 (stably express a 84 kDa rat GHR) cell lines were kindly provided by Dr. C. Carter-Su (University of Michigan Medical School, Ann Arbor, MI) from cell lines originally developed by N. Billestrup (Hagedorn Research Institute, Gentofte, Denmark). Mouse L cells (MLC) and W10 cells (MLC stably express the porcine GHR) were provided by Dr. X. Wang (Massachusetts General Hospital, Boston, MA).

#### *Rat adipocytes*

Rat adipocytes were prepared from rat epididymal and perirenal fat and used as our primary cell model in studies of GH action. Male rats of the CD strain were obtained from the Charles River Laboratories, Inc. (Kingston, NY) and studied when they attained body weights of 160-200 g. Epididymal and perirenal fat from several rats were pooled and minced for preparation of isolated adipocytes according to the procedure of Rodbell (145) as modified in this laboratory (78). After digestion for 20 min with 1 mg/ml collagenase (lot 143710, type A, Boehringer Mannheim Biochemicals, Indianapolis, IN)

in KRPG (Kreb's Ringer phosphate buffer contains 5.5 mM glucose) that contains 40 mg/ml bovine serum albumin (BSA: Metrix fraction IV, Reheis Chemical Co., Phoenix, AZ), the cells were washed four times in KRPG containing 10 mg/ml BSA, resuspended 1:3 (vol/vol) in the same buffer and incubated at 37 °C.

"Refractory cells" were prepared by stimulating freshly isolated cells with 100 ng/ml GH for 1 hour, followed by incubation in GH-free KRPG buffer (with 10 mg/ml BSA) for 2 more hours. "Sensitive cells" were obtained by incubating freshly isolated cells in GH-free KRPG buffer (with 10 mg/ml BSA) for 3 hours. Both freshly isolated and GH-pretreated cells are refractory to GH-induced insulin-like effects, whereas sensitive cells are responsive to GH-induced insulin-like effects.

#### *RNA extraction and Northern Blot analysis*

Northern blot analysis was used to examine GH regulation of CIS/SOCS mRNA expression. Total RNA was extracted from rat adipocytes using guanidinium thiocyanate-acid phenol (30). Adipose tissue or adipocytes were homogenized in GTC solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauryl sarcosine, 0.1 M 2-mercaptoethanol) (3 ml/g adipose tissue) using a Dounce homogenizer. After centrifugation at 10,000×g for 10 minutes at 4°C, the aqueous portion was mixed with 0.1 volume 2 M NaAc, pH 4.7, 1 volume phenol and 0.2 volume chloroform/isoamyl alcohol (24:1). After centrifugation the clear aqueous portion of the mixture was precipitated with isopropanol. The pellet was resuspended in GTC solution and further precipitated with 70% ethanol. The precipitated total RNA was re-dissolved in DEPC(diethyl pyrocarbonate)-treated water.

Total RNA was fractionated by electrophoresis through 1% agarose-formaldehyde gels and transferred and subsequently UV cross-linked onto GenePlus membrane (NEN Life Science Products, Boston, MA). Random primed,  $^{32}\text{P}$ -labeled probes were prepared from full-length cDNA inserts encoding CIS, SOCS1, SOCS2, SOCS3 and SOCS4 using a random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Membranes were pre-hybridized in pre-warmed Rapid-hyp buffer (Amersham Pharmacia Biotech Inc., Piscataway, NJ) for 30 minutes at  $65^{\circ}\text{C}$ , then hybridized with radiolabeled probes for 2.5 hours. Membranes were washed for 20 minutes in  $2\times\text{SSC}$  ( $20\times\text{SSC} = 3\text{ M NaCl}$ ,  $0.3\text{ M}$  sodium citrate),  $0.1\%$ (w/v) sodium dodecylsulfate (SDS) at room temperature followed with  $2\times 15$  minute washes in  $0.1\times\text{SSC}$ ,  $0.1\%$  SDS at  $65^{\circ}\text{C}$ . Membranes were then exposed to X-ray film with an intensifying screen at  $-70^{\circ}\text{C}$ .

#### *Cell culture and transfections*

Mouse L cells, CHO cells, HEK 293 cells and 293A cells were maintained in Dulbecco's Modified Eagle Medium with  $4.5\text{ g/L}$  glucose (DMEM high) (Gibco life technologies, Rockville MD) supplemented with  $10\%$  fetal bovine serum (Gibco life technologies, Rockville MD). Transient expression of proteins was accomplished using either the calcium phosphate precipitation method (adapted from (102)) or Superfect reagent from Qiagen (Santa Clarita, CA). Cells grown in  $100\times 20\text{mm}$  tissue culture dishes were transfected with  $10\text{ }\mu\text{g}$  plasmid DNA. For transfection by calcium phosphate precipitation, the cells were  $40\text{-}50\%$  confluent by the time of transfection. DNA precipitates were made by mixing eukaryotic expression vector with  $1\text{ml}$   $1\times\text{HEBS}$  ( $5\text{ g/L}$  HEPES,  $8\text{ g/L}$  NaCl,  $0.37\text{ g/L}$  KCl,  $0.125\text{ g/L}$   $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ ,  $1\text{ g/L}$  glucose, pH 7.1)



followed by dropwise addition of 50  $\mu$ l of 2.5 M  $\text{CaCl}_2$  solution. The precipitates were left at room temperature for 15 minutes and subsequently added dropwise to the dish containing the cells. The medium was removed after 4 to 16 hours. The transfected cells were washed once with phosphate buffered saline (PBS) and incubated in cell growth medium for 48 hours before harvesting. For transfection using Superfect reagent, cells were 60-80% confluent at the time of transfection. Plasmid DNA was first diluted with 300  $\mu$ l cell growth medium containing no antibiotics or serum and then mixed with 60  $\mu$ l Superfect reagent. After incubation at room temperature for 10 minutes, the mixture was diluted with 3 ml cell growth medium and added to PBS-washed cells. Three hours later, the medium containing DNA and transfection reagent was removed. The cells were washed with PBS, then incubated in cell growth medium.

#### *Cell stimulation and protein extraction*

Rat adipocytes were incubated in 1:3 KRPG buffer with 10 mg/ml BSA at 37 °C. hGH was added to the buffer to a final concentration of 500 ng/ml. Cells were incubated for 2 minutes or longer as indicated, washed with ice cold PBS/0.4 mM sodium orthovanadate, and lysed in 1:2 (vol/vol) cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 100 mM NaF, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 10 mM benzamidine, 10  $\mu$ g/ml aprotinin). After centrifugation at 12,000 $\times$ g for 10 minutes at 4°C, the supernatants were subjected to immunoprecipitation as described below.

Cultured cells were serum-starved in DMEM high/0.5% BSA for 16 hours, hGH was added directly to the medium at a final concentration of 500 ng/ml. Cells were incubated for 2 minutes or longer as indicated in the text, washed with ice cold PBS/0.4 mM sodium orthovanadate, and lysed in 1 ml cell lysis buffer. The lysed cells were then scraped off the dish. After centrifugation at 14,000×g for 20 minutes at 4°C, the supernatants were subjected to either immunoprecipitation or polyacrylamide gel electrophoresis (PAGE).

#### *Immunoprecipitation and Western Blotting*

Immunoprecipitation was used to enrich the protein of interest from cell lysates by its specific antiserum. It was also used to study the interaction of a particular protein with other molecules under appropriate condition, which was referred to as co-immunoprecipitation. The precipitates were then analyzed by Western blotting to examine the presence and/or the abundance of a particular protein by using an antiserum against this molecule. Practically, cell lysates were mixed and rotating with the appropriate antibody and 25 µl protein A agarose beads (1:4 suspension) overnight at 4°C. The immune complexes were then washed 4 times with TSA (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 0.025% NaN<sub>3</sub>)/0.1% Triton X-100. The proteins were released from the protein A agarose beads by boiling in Laemmli sample buffer (104) for 2 minutes and were resolved by SDS-PAGE prior to immunoblotting.

Proteins were denatured and separated by SDS-PAGE, transferred to PVDF-plus membrane (Micron Separation Inc., Westborough, MA). After blocking with 20% horse serum/TSA (for anti-phosphotyrosine) or 5% milk in washing buffer (10 mM Tris, pH

7.4, 150 mM NaCl, 0.2% Tween 20) for 1 hour, the membranes were immunoblotted with appropriate primary and secondary antibodies (diluted in washing buffer). Detection of the blots was achieved by ECL-plus reagents (Amersham Pharmacia Biotech Inc., Piscataway, NJ). For stripping and reprobing, the membranes were washed with TSA, followed by incubating in stripping buffer (60 mM Tris, pH 6.8, 2% SDS, 0.7% 2-mercaptoethanol) at 50 °C for 30 minutes. The stripped membranes were then rinsed with washing buffer and ready for reprobing.

*Immobilization of antibody to protein A agarose*

To avoid the interference of the light chain of immunoprecipitating antibody (~50 kDa) in Western blotting when detecting CIS or SOCS3 (29-40 kDa), the antisera used for immunoprecipitation were immobilized on protein A agarose beads using the crosslinking reagent dimethylpimelimidate (DMP). Briefly, protein A agarose beads were suspended in TSA, and CIS or SOCS3 antisera were added and allowed to bind at 4°C for 2 hours with gentle rocking. The agarose beads were then washed with TSA and 0.1 M sodium borate buffer, pH 9.0, and resuspended in sodium borate buffer. 0.2 M freshly prepared DMP was added and the mixture was incubated at 23°C for 30 minutes on a rocker. The reaction was stopped by centrifugation and aspiration of the supernatant. The agarose beads were then resuspended in 0.2 M ethanolamine to cap any partially reactive cross-linking reagent. The beads were washed with TSA and resuspended in TSA and ready to use.

### *Deglycosylation reaction*

Glycosidase F (Endo F) removes N-linked carbohydrate moieties from glycoproteins. In the deglycosylation reaction, immune complexes were boiled for 2 minutes in 15  $\mu$ l of 0.4% SDS, 1% 2-mercaptoethanol, mixed with 40  $\mu$ l of a solution containing 1% NP-40, 67 mM NaPi, pH 7.4, 2 mM sodium orthovanadate, protease inhibitor mixture and 2  $\mu$ l N-glycosidase F (0.043 unit/ $\mu$ l, Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubated at 37°C overnight. The reaction was stopped by addition of Laemmli sample buffer and boiling for 2 minutes.

### *Generation of Glutathione S-Transferase (GST)-CIS fusion protein and preparation of GST-CIS beads for pulldown assay*

A GST-CIS fusion protein was generated to precipitate (pulldown) CIS and CIS-associated proteins, in the same manner as the antibody to CIS. The Eco RI/Xba I fragment of pcDNA3/*myc*-CIS which contains the entire coding region sequence of CIS cDNA was digested and inserted into pBluescript vector to acquire appropriate restriction sites for cloning. The Eco RI/Nco I fragment was then excised from pBluescript/CIS and ligated to pGEX4T-3 digested by the same enzymes. The DNA junctions in pGEX4T-3/CIS so generated were confirmed by sequencing analysis. Expression of this construct produces a GST-CIS fusion protein with GST at the N-terminus of CIS protein and a thrombin cleavage site between GST and CIS sequences.

Fusion protein induction and affinity purification on glutathione Sepharose 4B beads (GST beads) were performed as suggested by the manufacturer. Briefly, bacterial cells were allowed to grow to  $OD_{600}=1.0$  at 30°C followed by induction with 0.1 mM

isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG) for 2 more hours. Cell lysates were prepared by resuspending cell pellets in PBS/1% Triton X-100 and sonication. The fusion protein was bound to 20  $\mu$ l GST beads (1:1 slurry in PBS) followed by extensive washing before mixing with cell lysates and incubation at 4°C overnight. To assess the bound GST-CIS fusion protein, after washing with cell lysis buffer 5 times, the fusion protein was dissociated and denatured in Laemmli sample buffer and resolved on SDS-PAGE. The fusion protein was examined by Coomassie blue staining and destaining or Western blotting.

*Affinity purification of p120 using immobilized GST-CIS*

GST-CIS fusion protein was prepared from 3 liters bacterial culture (enough to produce 6 mg GST-CIS) and bound to a 2 ml glutathione Sepharose 4B column. Cell lysates were prepared from  $3 \times 10^9$  GH-stimulated 293A<sup>1</sup> cells, and the lysates were allowed to pass through a prebound GST glutathione Sepharose 4B column at 4°C. The flow through was then passed through the prebound GST-CIS glutathione Sepharose 4B column. The columns were washed with 500 ml cell lysis buffer and eluted with 10 ml elution buffer (100 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione, 120 mM NaCl). The eluates were collected in 1 ml fractions and examined by SDS-PAGE followed by Coomassie blue staining and destaining to estimate the amounts of GST-CIS fusion protein in each fraction. The fractions that contained GST-CIS were combined and diluted with 50 mM Tris-HCl, pH 7.4 and concentrated to 1ml with Centricon 30 column (Millipore Corporation, Bedford, MA) to get rid of reduced glutathione. Samples were then added with SDS to 0.1% final concentration and boiled for 5 minutes to denature

proteins. The prepared sample was immunoprecipitated with PY99-agarose as a second purification step. The precipitates were boiled in Laemmli sample buffer and resolved on 7.5% SDS-PAGE and visualized by silver staining.

*Generation of the GHR with a thrombin cleavage site*

A thrombin cleavage site (Leu Val Pro Arg Gly Ser) was introduced into the extracellular domain of the GHR C-terminal to the WSXWS motif between amino acids 229-230 by overlapping PCR. The sequences of the oligonucleotides used are: P1: 5' GTT TGC CTG GGA TCC GTG 3', P2: 5' TCC CCT AGG TAC GAG TAC TTC ACT GAA CTC GCT G 3', P3: CTC GTA CCT AGG GGA TCA CTC CGT GTA ACG TTT CCT 3' and P4: ACT CCG AGG TAC CAT CA 3'. The underlined sequence encodes the thrombin cleavage site. In the first round of PCR, P1 and P2 were used as primers to synthesize the rGHR cDNA fragment bp678-927, while P3 and P4 were used as primers to synthesize fragment bp927-1424. The two products from the first round PCR were then allowed to anneal and served as a template for the second round of PCR using P1 and P4 as primers. After purification using QIAquick PCR purification kit (Qiagen, Santa Clarita, CA), the final PCR product was digested by Bam HI and Kpn I, and ligated to pcDNAIAmp-rGHR digested with the same two restriction enzymes. The sequence of the construct was confirmed by sequencing analysis. The expressed protein, GHR-T, generated from this construct can be cleaved by thrombin into 2 fragments: one composed of 233 residues and containing the majority of the extracellular domain, and the other containing the remaining 19 residues of the extracellular domain, the transmembrane domain and the intracellular domain.

### *Thrombin digestion reaction*

Immune precipitates or GST-CIS precipitates were resuspended 1: 1 in PBS with 0.02 u/ $\mu$ l human thrombin (Novagen, Madison, WI). After incubation at 37°C for 2 hours, the protein A beads or GST beads were pelleted by centrifugation and boiled in Laemmli sample buffer for 2 minutes. The released molecules were analyzed by SDS-PAGE and Western blotting.

### *Generation of His-tagged GHRs*

A 6 $\times$ histine sequence was placed on the C- or N-terminus of the GHR. C-terminal His-tagged rGHR was generated by adding a 6 $\times$ histine sequence to the 3' end just before the stop codon of GHR cDNA. In order to avoid mutation generated by synthesizing a long sequence by PCR, primers were chosen to only synthesize part of the GHR cDNA (from bp1125 to the stop codon). The primers used are: 5' GGA AGA TCT TCT CAA GGA 3' and 5' ATG GGC CCG CGG CCG CCTA ATG GTG ATG GTG ATG ATG CTG CAT GAT TTT GTT CAG 3'. The underlined sequence codes for the 6 $\times$ histine epitope tag. After PCR, the His-tagged sequence was digested with Bgl II and Apa I, and then ligated to pcDNAIAmp-rGHR digested with the same enzymes. N-terminal His-tagged rGHR was generated from the plasmid pRcCMV-NHisrGHR<sub>s</sub> which contains the cDNA for N-terminal His-tagged short isoform of rGHR (GHR<sub>s</sub>) previously generated. The sequence coding for 6 $\times$ histine was inserted into the cDNA after the signal sequence of rGHR<sub>s</sub> by overlapping PCR. Because the cDNA sequence of GHR<sub>s</sub> is the same as that of the extracellular domain of GHR, except that coding for the hydrophilic tail, NHisrGHR was generated by this cut-and-paste method. The rGHR

fragment 317-2549 was digested from pcDNAIAmp-rGHR with restriction enzymes Apa I and Bsu 36I, and was ligated to pRcCMV-NHisrGHR<sub>s</sub> that was digested with the same enzymes. The NHisrGHR so-generated has a 6×histine epitope tag at the N-terminus inserted just after the first amino acid, methionine. The DNA junctions of both CHisrGHR and NHisrGHR constructs was confirmed by sequencing analysis.

#### *GH binding assay*

Cells were serum starved overnight before stimulation with 500 ng/ml hGH (10% <sup>125</sup>I-hGH + 90% hGH) for 2 minutes. Cells were then lysed with cell lysis buffer and scraped off the culture dish. After centrifugation at 12,000×g for 10 minutes, the cell lysates were transferred to a fresh tube and hGH (10% <sup>125</sup>I-hGH + 90% hGH, from the same preparation as that used to stimulate cells) was added to a final concentration of 20 ng/ml. Co-immunoprecipitation of <sup>125</sup>I-hGH was achieved by adding Ab2941 (1:1000) or αCIS (1:500) and 25 μl (1:4 in TSA) protein A agarose to the lysate mixture and rotated at 4°C overnight. The precipitates were pelleted, and washed thoroughly, and counted by a γcounter. Cell lysate with normal serum or no antiserum added was used as blank to subtract nonspecific binding. To calculate receptor levels from the amount of bound GH, a 1:1 complex was assumed (15).



## RESULTS AND DISCUSSION

### Section I

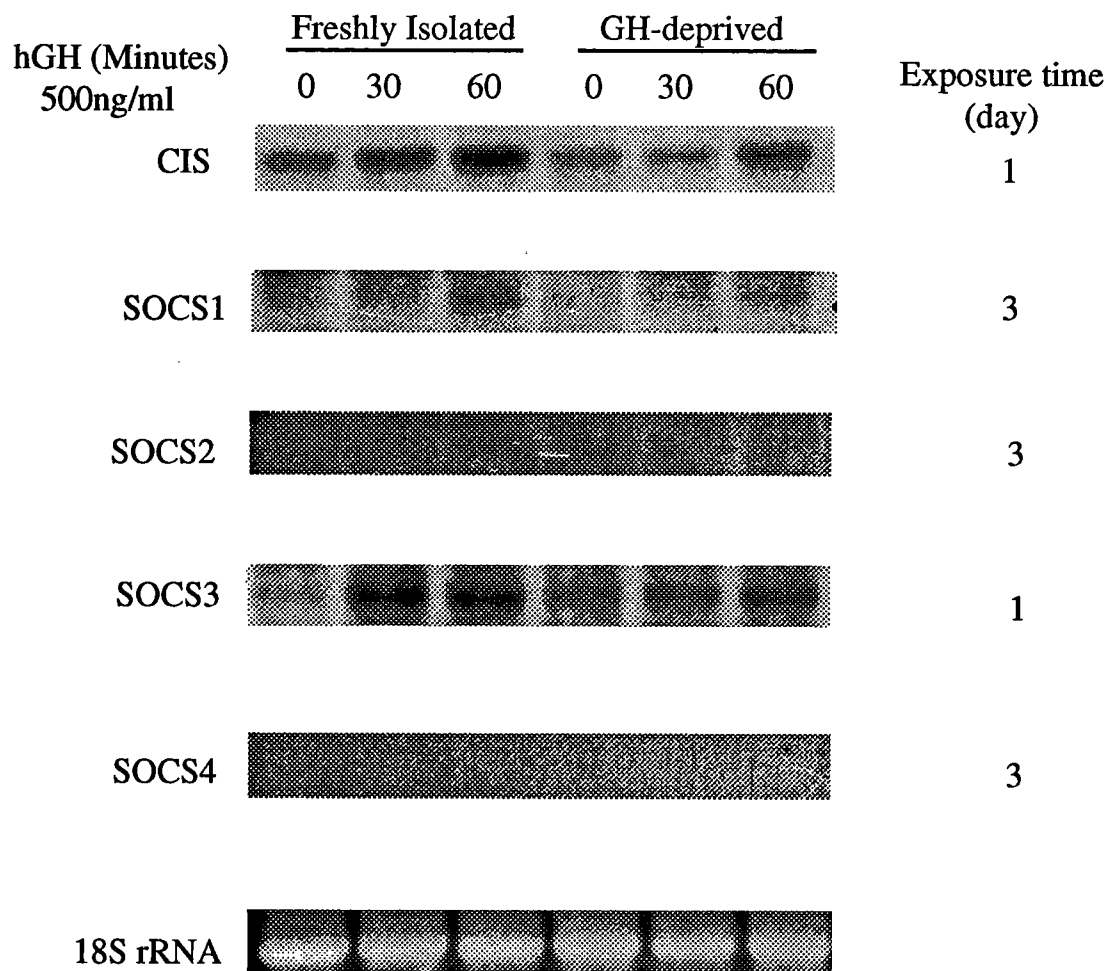
#### Involvement of CIS/SOCS Proteins in GH Actions in Rat Adipocytes

A number of cytokines induce CIS/SOCS gene expression, including GH. GH has been shown to induce CIS/SOCS mRNA expression in both 3T3-F442A cells and mouse hepatocytes (3). Adipose tissue is an important target of GH action and thus a good model to investigate the mechanism of GH effects. In order to determine the involvement and function, if any, of CIS/SOCS proteins in GH action in adipocytes, it is important to determine if GH regulates CIS/SOCS gene expression in these cells, and the molecules that might interact with CIS/SOCS proteins upon GH stimulation.

### Results

#### *Expression of CIS/SOCS genes in rat adipocytes*

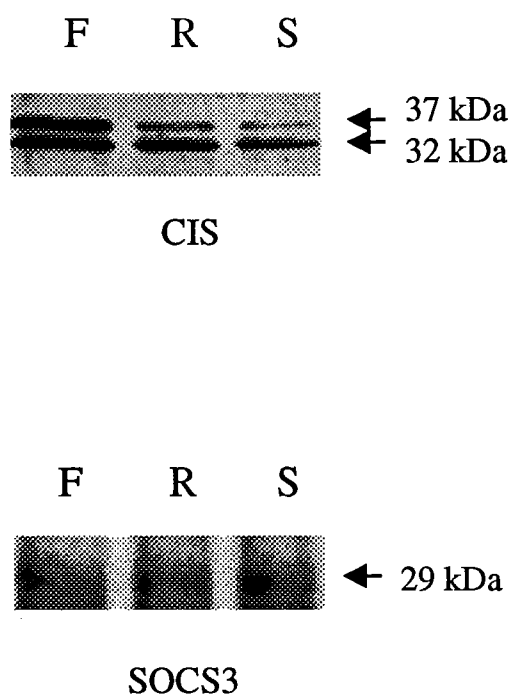
In freshly isolated adipocytes, a modest level of CIS mRNA was detected by Northern blot analysis (Figure 8). The expression of mRNAs for SOCS1 and SOCS3 was low, and mRNAs for SOCS2 and SOCS4 were undetectable in both freshly isolated and GH-deprived (sensitive) cells. Levels of mRNA for SOCS1 and CIS declined within 3 hours of incubation in the absence of GH (compare the basal levels from freshly isolated cells to those from sensitive cells). Upon the addition of GH, induction of CIS mRNA was apparent at 30 minutes, and increased even more at 60 minutes. The increase



**Figure 8.** GH up-regulates CIS/SOCS gene expression in rat adipocytes. Total RNA (15 µg/lane) was isolated from freshly isolated or GH-deprived (sensitive) adipocytes treated with hGH for the indicated times. RNAs were resolved by agarose gel electrophoresis, then transferred to nylon membranes. The membranes were hybridized with individual <sup>32</sup>P-labeled CIS/SOCS cDNA probes. The lengths of exposure time for autoradiography were as indicated and reflect signal strength. The results shown are representatives of three experiments.

in SOCS3 mRNA was dramatic at 30 minutes and the higher level was maintained for at least 60 minutes, and appeared to occur more rapidly in freshly isolated cells than in GH-deprived cells, in which the level of mRNA was greater at 60 minutes than at 30 minutes. The induction of SOCS1 became apparent by 60 minutes in both freshly isolated and GH-deprived cells, but the level was so low that it took longer exposure time for the autoradiography. In contrast, GH had no obvious effect on gene expression of SOCS2 and SOCS4. Notably, similar patterns of CIS gene expression were observed in both freshly isolated (refractory) and GH-deprived (sensitive) adipocytes after GH treatment. However, CIS gene expression levels were more prominent in freshly isolated adipocytes than in sensitive cells. From these results, it is evident that CIS, SOCS1 and SOCS3 are responsive to GH treatment in rat adipocytes.

Because the level of mRNA is not always indicative of protein expression, the amounts of CIS, SOCS1 and SOCS3 proteins in rat adipocytes were evaluated by Western blot analysis using antisera specific to these proteins. While it is difficult to find these proteins directly in whole cell lysates, CIS and SOCS3 were readily detectable after immunoprecipitation with  $\alpha$ CIS or  $\alpha$ SOCS3 antisera. However, the signal for SOCS3 was much weaker than that for CIS, so it took much longer exposure time to detect SOCS3, which is consistent with the low basal level of SOCS3 mRNA in adipocytes as shown in Figure 8. CIS was detected as two bands with molecular mass of 37 kDa and 32 kDa (Figure 9). It has been demonstrated in a transcription-translation experiment that the *in vitro* translated protein corresponds to the 32 kDa form, and the 37 kDa form is a ubiquitinated modification of CIS (188). SOCS3 protein has a molecular mass of about

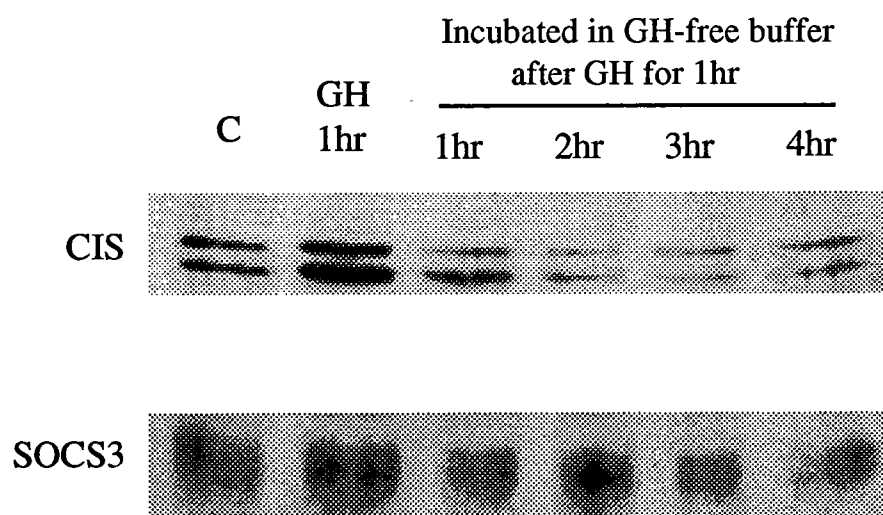


**Figure 9.** Detection of basal levels of CIS and SOCS3 proteins in rat adipocytes. Cell lysates from freshly isolated (F), refractory (R) or sensitive (S) adipocytes were prepared and immunoprecipitated with  $\alpha$ CIS or  $\alpha$ SOCS3 immobilized on protein A agarose beads. The samples were analyzed by SDS-PAGE followed by Western blotting with  $\alpha$ CIS or  $\alpha$ SOCS3 antiserum, respectively, and visualized by ECL plus detection system.

29 kDa. In agreement with the Northern blot data, CIS protein was more abundant in freshly isolated and refractory adipocytes than in the GH-deprived, sensitive cells, which is consistent with the up-regulation of CIS gene expression by GH. In contrast, SOCS3 levels remained relatively constant in freshly isolated, sensitive and refractory adipocytes. This suggests that there may be other transcriptional-translational regulation or protein degradation mechanisms involved to maintain a constant level of SOCS3 level even though its mRNA level was augmented in the presence of GH for 30 minutes. We were unable to examine SOCS1 protein expression, because it was not detected in the whole cell lysate by SOCS1 antibody and no SOCS1 antibody was available for immunoprecipitation. Considering its low mRNA level as shown by Northern analyses described above, it would not be surprising if SOCS1 is undetectable in adipocytes.

CIS and SOCS3 protein levels were further examined in consideration of GH's secretion pattern of one pulse every 3-4 hours. GH's pulsatile secretion pattern in male rodents was mimicked by challenging adipocytes with GH for 1 hour, followed by incubating in GH-free buffer for 4 more hours. CIS protein levels were dramatically increased by GH in the first hour, and declined to the basal level 2-3 hours later during incubation in GH-free buffer (Figure 10). In contrast, the amount of SOCS3 did not show any significant variation. However, both CIS and SOCS3 maintained detectable basal protein levels in adipocytes for at least 4 hours even in the absence of GH.

Together, these results demonstrated that GH up-regulates CIS and SOCS3 gene expression, suggesting that both CIS and SOCS3, but especially CIS, might be downstream mediators involved in GH action in rat adipocytes.

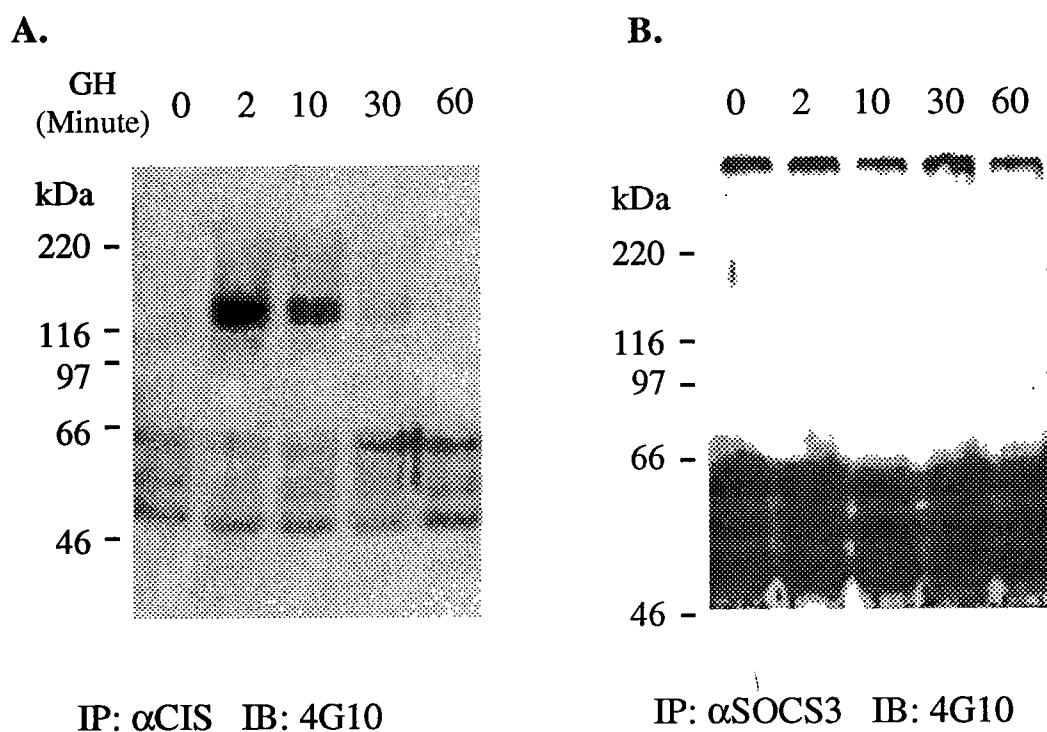


**Figure 10.** GH regulation of CIS and SOCS3 proteins in rat adipocytes. Freshly isolated adipocytes were incubated in buffer with 500 ng/ml hGH for 1 hour, followed by incubation in GH-free buffer for indicated length of time. The lysates were immunoprecipitated with immobilized  $\alpha$ CIS or  $\alpha$ SOCS3 and analyzed by SDS-PAGE followed by immunoblotting with  $\alpha$ CIS and  $\alpha$ SOCS3, respectively.

*A tyrosine phosphorylated 120 kDa protein co-immunoprecipitates with CIS following GH stimulation in adipocytes*

The existence of an SH2 domain in CIS/SOCS proteins suggests that these proteins may interact with tyrosine phosphorylated molecules. This interaction could be essential for exertion of their physiological effects. Candidate molecules that may bind these peptides were screened using a co-immunoprecipitation assay with CIS or SOCS3 as bait molecules. The precipitates were then analyzed by Western blotting. In lysates from GH-stimulated rat adipocytes, a highly tyrosine phosphorylated protein (or proteins) appeared to co-precipitate with CIS as detected by anti-phosphotyrosine antibody 4G10 (Figure 11A). This protein has an apparent molecular weight of approximately 120 kDa, which is similar to that of the tyrosine phosphorylated GHR. This protein is referred to as p120. The abundance of tyrosine phosphorylated p120 peaked at 2 minutes, subsided by 10 minutes, decreased to a very low level by 30 minutes post GH treatment, and disappeared by 60 minutes. The progressive attenuation of the p120 band could be due to either the change of its phosphorylation status, or its association with CIS. On the other hand, no GH-responsive phosphorylated protein was detected that interacts with SOCS3 by co-immunoprecipitation (Figure 11B).

As described previously, CIS associates with the tyrosine phosphorylated Epo receptor or the IL-3 receptor  $\beta$  chain following stimulation with Epo or IL-3, respectively (205). GHR belongs to the same receptor family as the Epo and the IL-3 receptors. Therefore, it is likely that CIS might associate with tyrosine phosphorylated GHR following GH stimulation. Furthermore, p120 shares the same apparent molecular weight



**Figure 11.** A tyrosine phosphorylated 120 kDa protein (p120) co-immunoprecipitates with CIS following GH treatment in adipocytes. Freshly isolated rat adipocytes were prepared and treated with hGH for the indicated times. The cells were then lysed and the lysates were subjected to immunoprecipitation with  $\alpha$ CIS (A) or  $\alpha$ SOCS3 (B). The precipitated proteins were analyzed by SDS-PAGE followed by Western blotting probed with anti-phosphotyrosine 4G10, and visualized by ECL plus detection system.



on SDS-PAGE with the GHR and both proteins are tyrosine phosphorylated in GH-stimulated cells. This evidence supports the hypothesis that p120 may be the GHR.

To test this hypothesis, the membrane shown in Figure 11A was stripped and reprobed with Ab2941, an antiserum that was raised against the intracellular domain of the GHR. Ab2941 did not detect any signal at the position of 120 kDa, nor did another GHR antiserum BB74 that was raised against the extracellular domain of the GHR. The same result was obtained when proteins in lysates prepared from  $2 \times 10^6$  adipocytes were immunoprecipitated with  $\alpha$ CIS and analyzed directly by immunoblotting with Ab2941 or BB74. Subsequently, other proteins with similar electrophoretic mobility (or molecular weight) were tested by Western blot analysis using corresponding antibodies. The tested proteins included the IL-3 receptor  $\beta$  chain, gp130, JAK2, Sirp (172), FAK, c-cbl and P130<sup>CAS</sup> (212). However, none of these antibodies recognized p120. Thus we failed to identify p120 by immunoblotting with available antibodies.

There could be several explanations for this result: First, p120 is distinct from the GHR or any of those proteins mentioned above. Secondly, p120 is the GHR or one of those proteins mentioned above, but the GHR antibodies or the other antibodies used were much less sensitive in immunoblotting than 4G10. Thirdly, p120 is the GHR or one of those proteins and the detecting antibody is no less sensitive than 4G10, but because p120 is phosphorylated on multiple tyrosines, it has many more antigenic sites and hence is more easily detected by  $\alpha$ PY.

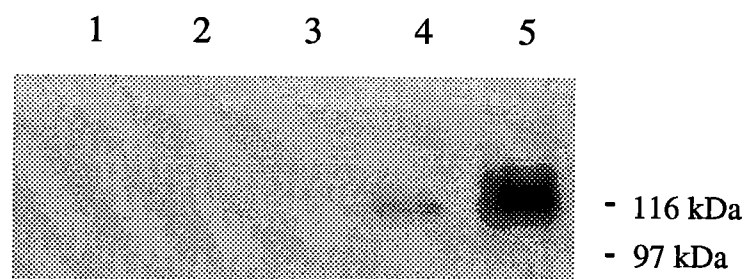
To test the possibility that the failure of GHR antibodies to detect p120 is due to lower sensitivity of GHR antibodies than the  $\alpha$ PY, 4G10, the antibodies were tested in

parallel. We prepared immunoprecipitates from different amounts of lysates of GH-stimulated adipocytes using affinity purified Ab2941 as the immunoprecipitation antibody. The precipitates were analyzed on duplicate membranes probed with either Ab2941 or 4G10 (Figure 12). Assuming that using GH at a concentration (500 ng/ml) that was 25 times higher than the  $K_d$  for the receptor to stimulate the cells is likely to result in all the GHR in cells being tyrosine phosphorylated ( $K_d=20$  ng/ml, (78)), Ab2941 showed the same, if not greater, sensitivity in recognizing GHR compared to that of 4G10. These data suggest that the failure to detect p120 by Ab2941 is not due to its lower sensitivity, but rather that p120 may not be GHR or that only a small fraction of highly phosphorylated GHR protein associates with CIS.

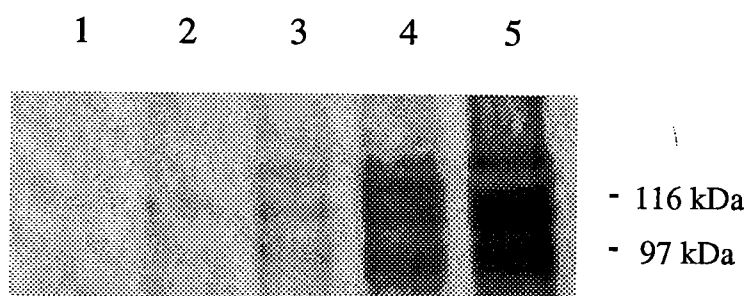
The third possibility could be addressed by using a larger sample size or different system to facilitate efficient detection by immunoblotting. However, even when lysates from a ten times larger sample, i.e.,  $4 \times 10^7$  GH-stimulated adipocytes were used for immunoprecipitation of p120, no specific signal at the position corresponding to 120 kDa was detected by immunoblotting with GHR antibodies, whereas again p120 was easily detected by 4G10.

#### *CIS protein complexes with the GHR*

The similar molecular weight and tyrosine phosphorylation status of p120 and the GHR complicated the study of the relationship of CIS to the GHR. In order to distinguish p120 from the GHR, a sequential immunoprecipitation procedure was employed to examine the interaction of CIS to the GHR and to p120. Whole cell lysates



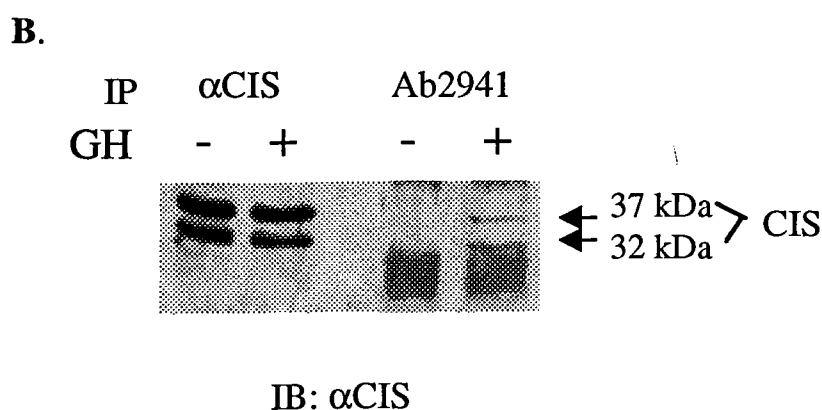
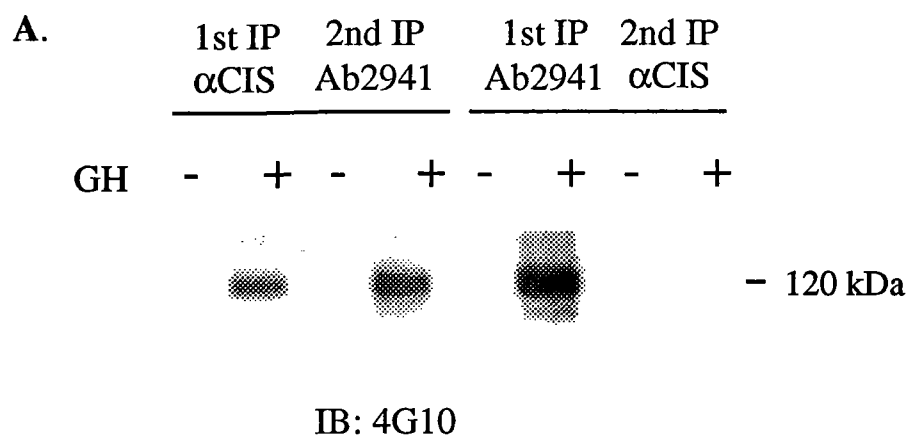
IB: 4G10



IB: Ab2941

**Figure 12.** Comparison of the immunoblotting sensitivities of Ab2941 and 4G10. Lysates from GH-stimulated adipocytes (Lane 1, 25  $\mu$ l; Lane 2, 50  $\mu$ l; Lane 3, 100  $\mu$ l; Lane 4, 250  $\mu$ l; Lane 5, 500  $\mu$ l) were immunoprecipitated with Ab2941. The precipitates were loaded on 2 gels for SDS-PAGE, followed by immunoblotting with 4G10 or Ab2941.

prepared from freshly isolated adipocytes incubated with or without GH were divided equally into two groups. Lysates of one group were first immunoprecipitated with  $\alpha$ CIS. The supernatants were collected and further immunoprecipitated with  $\alpha$ GHR (Ab2941). Lysates of the other group were first immunoprecipitated with Ab2941, and then with  $\alpha$ CIS. All precipitates from both sets of reactions were subjected to SDS-PAGE followed by Western blot analysis with 4G10 antibody. The results are shown in Figure 13A. In the first group, p120 was detected by 4G10 in the first precipitation with  $\alpha$ CIS as observed previously. Even after preclearing with  $\alpha$ CIS, tyrosine phosphorylated GHR was still detectable by 4G10 in the subsequent precipitation with Ab2941. On the other hand, in lysates first precipitated with  $\alpha$ GHR, a much stronger GHR signal was detected. Strikingly, no p120 signal was detected in the subsequent precipitation with  $\alpha$ CIS. This result indicated that Ab2941 brought down the GHR as well as p120 from the cell lysates in the first step precipitation, which resulted in the absence of p120 in samples immunoprecipitated with  $\alpha$ CIS. In other words, it is likely that p120 either is the GHR or is a protein that forms a complex with a small fraction of the GHR in cells stimulated with GH. No matter what relationship p120 has with the GHR, this experiment demonstrated that CIS must interact with the GHR directly or indirectly. However, CIS only interacts with and hence co-precipitates with a small fraction of the phosphorylated GHR, which might explain why much of the GHR was still left to be precipitated with Ab2941 after preclearance with  $\alpha$ CIS (Figure 13A). The p120 signal detected in the precipitates brought down with  $\alpha$ CIS was much weaker than the GHR signal from the  $\alpha$ GHR precipitates, suggesting the phosphorylated GHR is in great excess compared to



**Figure 13.** CIS interacts with the GHR. **A.** Freshly isolated adipocytes were treated with 500 ng/ml hGH for 2 minutes. Cell lysates were then immunoprecipitated either with  $\alpha$ CIS or Ab2941 at 4°C overnight. The supernatants were transferred to fresh tubes and further immunoprecipitated with Ab2941 or  $\alpha$ CIS, respectively. The precipitated proteins were analyzed by Western blotting with 4G10. **B.** Adipocyte lysates were precipitated  $\alpha$ CIS or Ab2941. The precipitates were analyzed by immunoblotting with  $\alpha$ CIS. The results shown were representatives of three independent experiments.

the phosphorylated p120 or that Ab2941 brings down other tyrosine phosphorylated 120 kDa proteins in addition to the GHR. Furthermore, the amount of p120 precipitated by  $\alpha$ CIS and the amount of GHR precipitated by  $\alpha$ GHR in the first group, when combined, approximate the amount precipitated by  $\alpha$ GHR alone. These results suggest that CIS, p120 and the GHR are components of a complex present in rat adipocytes following GH stimulation.

If CIS, p120 and the GHR are part of a complex,  $\alpha$ GHR should be able to precipitate CIS as well. Thus rat adipocyte lysates were subjected to immunoprecipitation with Ab2941 immobilized on protein A agarose. The CIS protein that co-precipitated was detected by immunoblotting with  $\alpha$ CIS. As expected,  $\alpha$ GHR did precipitate CIS proteins, both 37 kDa and 32 kDa forms (Figure 13B), though they are only a small fraction of the total CIS protein in the lysates as precipitated and detected by  $\alpha$ CIS. No differences in the binding of the GHR to either the 37 kDa or the 32 kDa forms of CIS were observed. This result confirmed that CIS has direct or indirect contact with GHR. However, apparently the amount of GHR that interacts with CIS was too small to be detected by GHR antibodies.

#### *p120 is a glycoprotein*

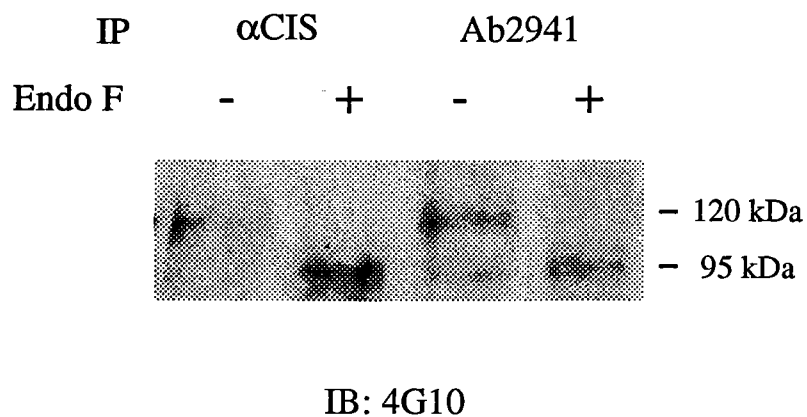
To gain further insight about the nature of p120, we examined its glycosylation status. The broad, fuzzy shape of the p120 band shown in Western blot analysis suggested that it might be glycosylated. N-glycosidase F (Endo F) removes N-linked carbohydrate from glycosylated proteins. Therefore we prepared p120 by  $\alpha$ CIS

immunoprecipitation from lysates of GH-stimulated adipocytes and digested the precipitates with Endo F. Endo F digestion analysis shifted the p120 band to ~ 95 kDa on SDS-PAGE (Figure 14), indicating that p120, like the GHR, is a glycoprotein. It has been previously demonstrated that Endo F treatment of the GHR reduced its molecular weight from 120 kDa to about 95 kDa (101). This correlation in shift of molecular weight by Endo F treatment of p120 and GHR indicated that they may have similar amounts of N-linked carbohydrate and that p120 might be a transmembrane protein.

Sirp is another glycosylated protein that is also tyrosine phosphorylated upon GH stimulation and has an apparent molecular mass of 120 kDa (172). Thus Sirp could be a good candidate other than GHR for p120. However, anti-Sirp did not recognize p120. Furthermore, in contrast to p120, Sirp maintains a high basal level of tyrosine phosphorylation in adipocytes even without GH stimulation (Figure 15) and after deglycosylation Sirp migrates with an electrophoretic mobility corresponding to 65 kDa (172), whereas the apparent molecular weight of deglycosylated p120 is 95 kDa. Thus p120 could not be Sirp.

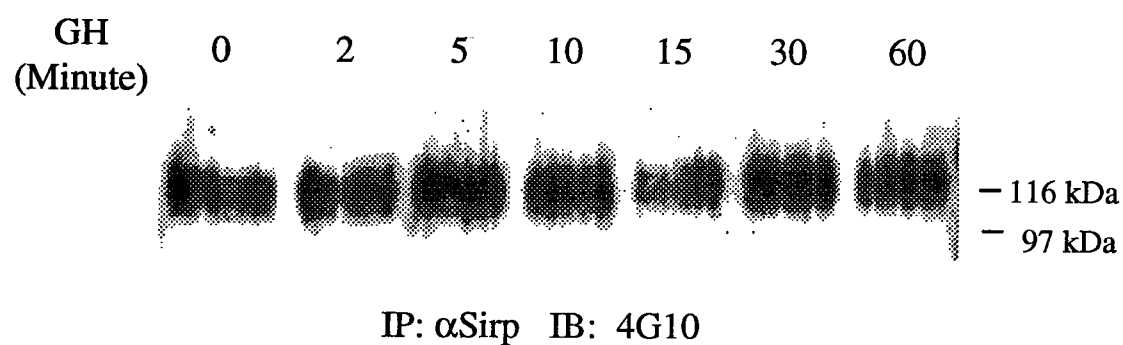
## Discussion

Results reported here indicated that GH regulates CIS, SOCS3 and SOCS1 mRNA expressions in rat adipocytes. A tyrosine phosphorylated protein (p120) was found to co-precipitate with CIS following GH stimulation, but no tyrosine phosphorylated proteins were found to co-precipitate with SOCS3. p120 is glycosylated and appears to have the same apparent mass of its N-linked carbohydrate component as



**Figure 14.** p120 is a glycoprotein. Freshly isolated rat adipocytes were stimulated with 500 ng/ml hGH for 2 minutes. p120 and GHR were precipitated from the lysates with  $\alpha$ CIS and Ab2941, respectively. The precipitates were then divided into two halves. One half was digested with Endo F, the other half serves as control. The samples were then released from protein A beads and analyzed by SDS-PAGE followed by immunoblotting with 4G10.





**Figure 15.** GH regulation of Sirp phosphorylation in adipocytes. Freshly isolated adipocytes were challenged with GH for indicated lengths of time. Lysates were prepared from these cells and immunoprecipitated with  $\alpha$ Sirp. The precipitates were analyzed by SDS-PAGE followed by immunoblotting with 4G10.

that of the GHR. It appeared that three proteins, CIS, p120 and the GHR form a complex upon GH stimulation of the adipocytes.

It is puzzling that GHR antibodies did not detect any GHR signal from the immunoprecipitate of  $\alpha$ CIS even though CIS was detected in the immunoprecipitates of Ab2941. Technically, two aspects can account for this discrepancy. On the immunoprecipitation step, the efficiency of  $\alpha$ CIS may not be as good as that of Ab2941. Thus Ab2941 might bring down more CIS compared to the amount of the GHR that precipitated with  $\alpha$ CIS. Even if  $\alpha$ CIS and Ab2941 have similar efficiencies for immunoprecipitation, on the immunoblotting step, 4G10 and Ab2941 might have different sensitivities in detecting corresponding proteins. Though there may be multiple epitopes in the intracellular domain of the GHR that are recognized by Ab2941, many of them may be recognized with low avidity. The conclusion of no less sensitivity of Ab2941 in detecting the GHR compared to 4G10 stands only if all the GHR in adipocytes precipitated by Ab2941 is tyrosine phosphorylated, an assumption that is still unverified. Furthermore, the signal from the GHR precipitated by  $\alpha$ CIS would be much stronger if CIS selectively binds to the GHR molecules that have multiple tyrosines phosphorylated. Therefore, we cannot rule out the possibility that GHR is one component of p120.

Among the five members of the CIS/SOCS family, GH induces expression of the mRNAs only of CIS, SOCS3 and SOCS1 in adipocytes. The preferential induction of CIS and SOCS3 by GH suggests that these two proteins might be the representatives of the family that are subject to regulation by GH in the adipocytes, and that they might be involved in GH action, possibly to negatively regulate GH signaling. Co-

immunoprecipitation studies showed that CIS is the only CIS/SOCS protein that associates with a detectable tyrosine phosphorylated protein in a GH-dependent way. This is not surprising considering that CIS is more abundant in fat tissue than in other tissues, and in fat tissue the CIS mRNA level is higher than those of SOCS2 and SOCS3 as revealed by the tissue distribution study by Tollet-Egnell and colleagues (182).

CIS was the first member in the CIS/SOCS family to be identified. It was cloned originally as an immediate-early gene that was induced by IL-3 and Epo (205). CIS associates with EpoR or IL-3R in response to Epo or IL-3 stimulation, respectively. Since the GHR belongs to the same superfamily as EpoR and IL-3R, it would be reasonable to assume that CIS might interact with the GHR, in other words, the p120 protein is the GHR. However, despite the findings that these two proteins share a similar electrophoretic mobility shift after deglycosylation and are both tyrosine phosphorylated after GH stimulation, GHR antibodies failed to recognize p120. Neither did several other antibodies that were described previously recognize the p120 band. Therefore, the identity of p120 remains unknown so far.

Studies using the intracellular domain of the GHR fused to glutathione S-transferase (GST) revealed that the ability of CIS/SOCS proteins to bind to the GHR varies among individual proteins in the family (83). SOCS1 interacts with both the non-tyrosine phosphorylated and the tyrosine phosphorylated GHR, whereas the interaction of CIS, SOCS2 and SOCS3 requires the tyrosine phosphorylation of the GHR (83), and SOCS6 does not bind to the GHR fusion protein at all (142). However, in these experiments, the GHR fusion proteins were tyrosine phosphorylated in bacteria by *elk*

kinase instead of JAK2. It is not clear if the tyrosines that are phosphorylated in bacteria are the same as those phosphorylated by JAK2 in eukaryotic cells. Therefore, one cannot necessarily predict that the same interaction will occur in physiologically activated cells. In the present study, we could detect no tyrosine phosphorylated protein that co-precipitates with SOCS3, including the tyrosine phosphorylated GHR, even though SOCS3 mRNA expression is induced by GH. As for the tyrosine phosphorylated p120 that co-precipitates with  $\alpha$ CIS, it has not been clarified yet whether it is the GHR or a novel protein. Nevertheless, the formation of the complex containing CIS, p120, GHR and perhaps some other proteins following GH stimulation implied that among the CIS/SOCS proteins, CIS protein could be the one that is involved proximately in GH signaling in rat adipocytes. No matter what the identity of the p120 protein, the strong and rapid association of CIS with p120 suggests that p120 might be an important mediator for the function of CIS in GH signaling pathway.

GH up-regulates the mRNA level of both CIS and SOCS3 in adipocytes. However, while changes in the protein level of CIS in adipocytes also reflects GH stimulation, protein levels of SOCS3 remained relatively constant in the presence or absence of GH. This finding implies that CIS and SOCS3 are under different transcriptional/translational regulation in adipocytes. The significance of such regulation in adipocytes is unclear. Interestingly, it was revealed recently that SOCS1 is strongly repressed at the level of translation initiation (77). Thus transcriptional/translational regulation could be an another mechanism to regulate CIS/SOCS protein expression. In addition, no tyrosine phosphorylated partner for SOCS3 was discovered upon GH

stimulation of adipocyte. The stable and low protein level of SOCS3 might imply that it is an unessential regulator of GH action in this particular type of cells. This finding is different from what was reported for SOCS3 in cultured cells. SOCS3 was proposed as the major regulator of GH signaling based on the observations that its mRNA is preferentially induced by GH in 3T3-F442A cells and mouse liver, that it inhibits GH-induced STAT5 activity in CHO cells and HEK 293 cells, and that it is the only protein in the family that inhibits JAK2 activity by binding to the tyrosine phosphorylated GHR in HEK 293 cells (83). We suggest the discrepancy between our observation and what was reported for SOCS3 may be explained as arising from the difference between adipocytes and cultured cells, and/or from the expression levels of the GHR in the cultured cells which modify the stoichiometry relationship between the GHR and SOCS3. Adipocytes are terminally differentiated primary cells that are metabolically active and do not proliferate. In primary adipocytes, there is no clonal expansion, or at least clonal expansion is not the main effect of GH, while mitogenesis is one of the main effects of GH in cultured cells. SOCS3 might be more important for the inhibition of the mitogenic effects of GH, whereas CIS might be more involved in termination of the metabolic effects of GH. Therefore, individual CIS/SOCS proteins might be engaged in different pathways of GH signaling.

## Section II

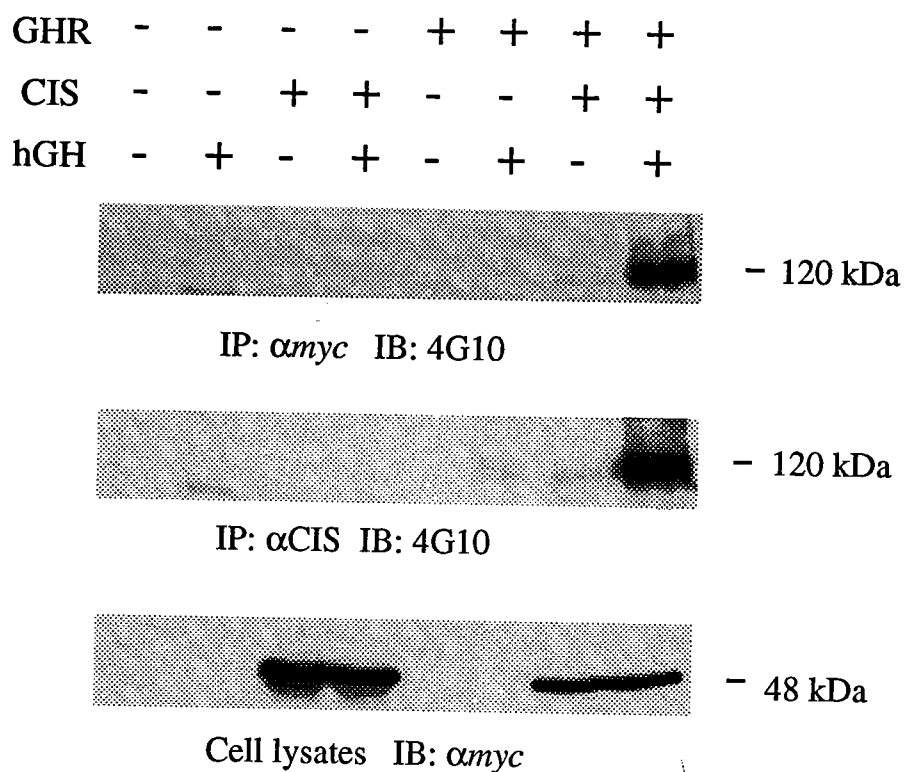
### CIS Interacts with the Tyrosine Phosphorylated GHR

In the previous section, it was shown that GH up-regulates gene expression of CIS/SOCS proteins in rat adipocytes. A tyrosine phosphorylated glycoprotein, p120, was found to co-precipitate with  $\alpha$ CIS, suggesting that CIS, p120 and GHR form a complex in adipocytes following GH stimulation. In order to study the physiological function of CIS in GH actions, it is important to know what molecules CIS interacts with. The strong association of CIS with p120 makes it imperative to characterize p120. However, rat adipocytes, as terminally differentiated cells, are much harder to manipulate than immortalized cultured cells. Therefore, I turned to cultured cells to seek ways to identify the mysterious p120 and determine if it is the GHR or a novel protein.

## Results

### *Association of p120 and CIS is dependent on GH/GHR*

To further examine the relationship of p120 to the GHR, the co-immunoprecipitation experiment was carried out in cultured mouse L cells (MLC) which express no or, at least non-detectable levels of endogenous GHR. MLC or MLC that stably express porcine GHR (196) were transiently transfected with a plasmid containing *myc*-tagged CIS cDNA. Both  $\alpha$ CIS and  $\alpha$ *myc* antibodies precipitated a tyrosine phosphorylated 120 kDa protein from lysates prepared from cells stimulated with GH, but only in samples from cells that express the GHR (Figure 16). This result confirmed



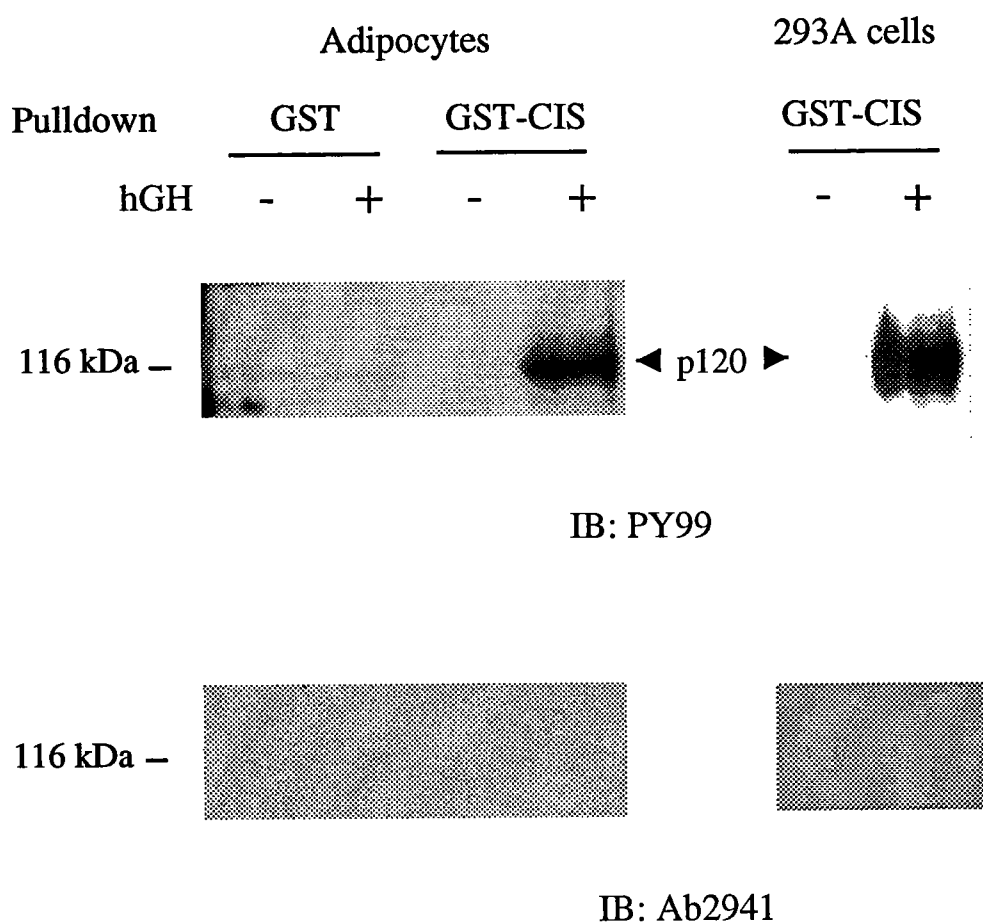
**Figure 16.** The co-immunoprecipitation of p120 with  $\alpha$ CIS is dependent on GHR. Parental MLC(no detectable GHR) and W10 (MLC stably expressing pGHR) cells were transiently transfected with either plasmid vector or a plasmid with *myc*-tagged CIS cDNA by calcium phosphate precipitation. Forty-eight hours after transfection, cells were treated with 500 ng/ml hGH for 2 minutes. Cell lysates were then collected and immunoprecipitated with either  $\alpha$ myc (upper panel) or  $\alpha$ CIS antibody(middle panel), followed by Western blotting with 4G10. The transfection efficiency was monitored by detecting CIS protein levels in cell lysates by HRP- $\alpha$ myc (lower panel). The results shown are representatives of three independent experiments.

the association of CIS with p120 in MLC, indicating that it is a phenomenon observed not only in primary rat adipocytes, but also in cultured cells. This experiment demonstrated that the association of CIS with p120 depends on the presence of the GHR on the cell surface. Furthermore, since p120 was only detected in GH stimulated cells, it is likely that CIS interacts with phosphorylated p120. However, because our ability to detect the 120 kDa protein that co-precipitates with CIS depends on its reaction with  $\alpha$ PY, we cannot rule out the possibility that unphosphorylated p120 also binds to CIS even in the absence of GH stimulation.

*Pulldown of p120 protein by bacterial-produced GST-CIS fusion protein*

To introduce an alternative way to study p120, and also to solve the problem of the limited supply of  $\alpha$ CIS, a plasmid construct for GST-CIS fusion protein was generated and transformed into bacteria cells to allow the production of a GST-CIS fusion protein. The bacterial-produced fusion protein binds to GST beads and can be used to affinity purify CIS interacting molecule(s) from mammalian cell lysates. First, the efficacy of GST-CIS to precipitate (pulldown) p120 was examined. Lysates from GH-stimulated rat adipocytes (500 ng/ml for 2 minutes) were incubated with immobilized GST-CIS. After extensive washing, the precipitates were analyzed by immunoblotting with  $\alpha$ PY. As expected, the  $\alpha$ PY recognized a 120 kDa protein in the precipitates formed with GST-CIS beads (Figure 17, upper panel) just as with  $\alpha$ CIS, suggesting the CIS moiety produced as a GST-CIS fusion protein retains its natural capability to associate with p120. This result not only demonstrated the feasibility of this approach to





**Figure 17.** p120 co-precipitates with bacterial-produced GST-CIS fusion protein. The GST and GST-CIS fusion proteins were produced as described in **MATERIALS AND METHODS**. The proteins were then immobilized on GST beads and mixed with cell lysates from adipocytes or 293A cells. The precipitates were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies.

pulldown p120 from GH stimulated lysates (to replace the  $\alpha$ CIS antibody), but also confirmed the previous results of the p120/CIS interaction from a different aspect. Furthermore, the 120 kDa band precipitated (from  $2 \times 10^6$  adipocytes) by GST-CIS was still not recognized by  $\alpha$ GHR (Figure 17, lower panel). Similar results were also observed in 293A cells (HEK 293 cells stably expressing the rat GHR) using this pulldown procedure (Figure 17).

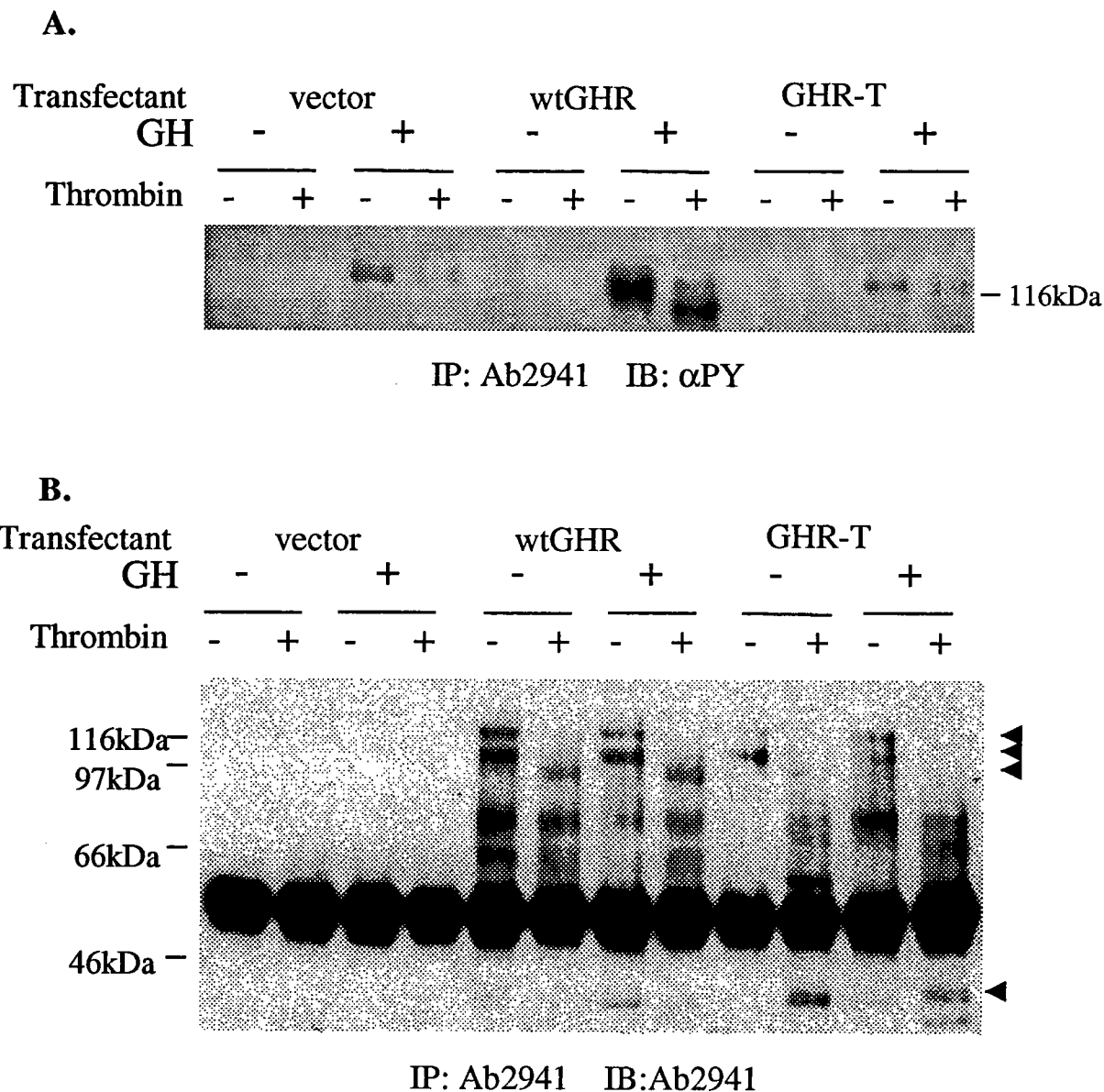
*Both p120 and rat GHR can be digested by thrombin*

Because the GHR and p120 have a similar electrophoretic mobility, the GHR may contribute to the phosphotyrosine signal even though it is present in an amount below the threshold of detection by Ab2941. The same electrophoretic mobility shared by both p120 and the GHR on SDS-PAGE became one of the difficulties encountered in attempting to differentiate signal arising from these two proteins. Therefore, I sought ways to alter the size of the GHR, that is, to make it either bigger or smaller. A thrombin cleavage site was introduced into the extracellular domain of the rat GHR near the transmembrane domain, C-terminal of the WSXWS motif. Thrombin should cleave this molecule into two fragments with electrophoretic mobilities of approximately 80 kDa and 40 kDa. The 80 kDa fragment contains the majority of the glycosylated extracellular domain, while the 40 kDa fragment has the remaining 19 residues of the extracellular domain, the transmembrane domain and the intracellular domain. Because only the intracellular domain has tyrosines that can be phosphorylated upon GH stimulation, thrombin digestion should shift the phosphorylated GHR from 120 kDa to 40 kDa when

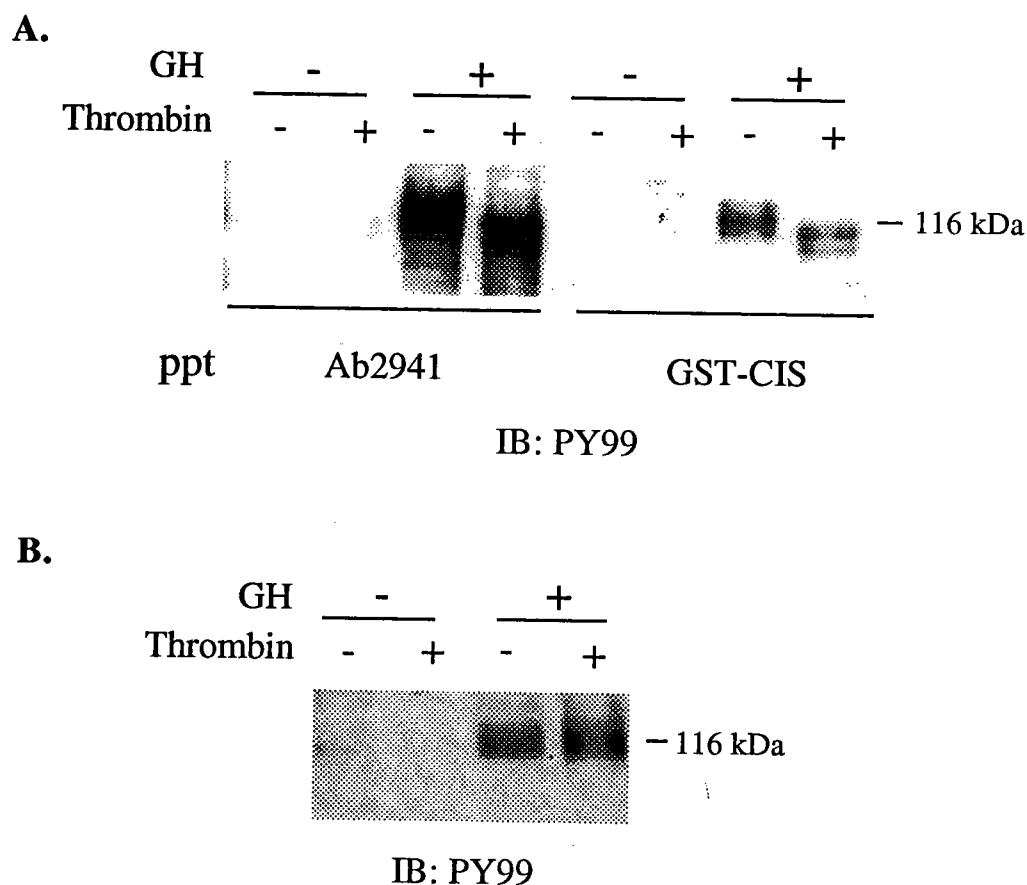
detected with either PY99 or Ab2941 which was raised against the intracellular domain of GHR. Thus, a plasmid construct containing wild type (wt) GHR or GHR with a thrombin cleavage site (GHR-T) was transiently transfected into HEK 293 cells. After GH stimulation, the cells were lysed and the lysates were precipitated with Ab2941 or GST-CIS. After incubation with thrombin, the precipitates were then analyzed by Western blotting with either PY99 or Ab2941.

In cells expressing GHR-T, little phosphorylated GHR could be detected by PY99, except for the weakly expressed endogenous GHR (Figure 18A), although there was an appreciable amount of GHR-T detectable by Ab2941 (Figure 18B). This suggested that insertion of the thrombin cleavage site in the extracellular domain either interferes with GH binding, or significantly changes the conformation of GHR in the membrane proximal region, so that little signal could be transduced into the cells, and thus JAK2 could not be efficiently recruited or activated to phosphorylate GHR-T.

Though the experiment did not work as expected, surprisingly, thrombin digestion shifted both the rat GHR and p120 from 120 kDa to 110 kDa (Figure 19A). Sequence analysis of the rat GHR revealed that there is a 3 amino acid sequence (amino acids 30-32) near the N-terminus that can be cleaved by thrombin. This thrombin cleavage site is present in GHR sequences from rat (Gly Lys Ala), mouse (Gly Lys Ala) and rabbit (Gly Arg Ala), but not in human, porcine, sheep or bovine (Table 1). Thus when overexpressed in HEK 293 cells, porcine GHR did not appear to be digested by thrombin (Figure 19B). Also, the weakly expressed endogenous human GHR in HEK 293 cells was unaffected by thrombin (Figure 18A). The presence of such a thrombin cleavage site



**Figure 18.** Insertion of a thrombin cleavage site severely affected GH-stimulated GHR phosphorylation. 293 cells were transiently transfected with plasmids pcDNA3/rGHR (wtGHR), pcDNA3/rGHR-T (GHR-T) or vector by Superfect reagent. Forty-eight hours after transfection, the cells were treated with GH for 2 minutes. Proteins immunoprecipitated with Ab2941 from cell lysates were digested with thrombin, then analyzed by SDS-PAGE followed by immunoblotting with PY99 (A) or Ab2941 (B). The arrows indicate the positions of GHR before and after thrombin digestion. The results shown here are representative of four experiments.



**Figure 19.** Both rat GHR and p120, but not porcine GHR can be digested by thrombin.

**A.** GHR and p120 were precipitated from lysates of GH-stimulated 293A cells by Ab2941 or GST-CIS. After digested with thrombin, the immune complexes were analyzed by SDS-PAGE followed by immunoblotting with PY99. The results shown here are representatives of four experiments. **B.** HEK 293 cells were transiently transfected with a plasmid containing the cDNA encoding the porcine GHR. The GHR was precipitated from lysates with Ab2941, digested with thrombin and analyzed as described above. The results represent two experiments.

in only some species is both interesting and puzzling, but its significance, if any, is unknown. Cleavage by thrombin should shorten the rat GHR by 14 residues, and thus reduce the apparent molecular mass by only about 1.7 kDa. There is no potential glycosylation site within or close to these 14 residues (Table 1). However, thrombin cleavage reduced the apparent molecular weight of the GHR by about 10 kDa as shown on SDS-PAGE, suggesting the existence of some covalently bound factor to the N-terminus of the rat GHR or some configuration that produces anomalous mobility on SDS-PAGE.

**Table 1.** The GHR sequences (amino acids 1-50) from different species

Species	GHR Sequences					References
Rat	MDLWRVFLTL	ALAVSSDMFP	GSGATPATL <b>G</b>	<b>K</b> ASPVLRIN	PSLRESSSGK	(118)
Mouse	MDLCQVFLTL	ALAVTSSSTFS	GSEATPATL <b>G</b>	<b>K</b> ASPVLRIN	PSLGTSSSGK	(163)
Rabbit	MDLWQLLLTV	ALAGSSDAFS	GSEATPATL <b>G</b>	<b>R</b> ASESVQRVH	PGLGTNSSGK	(107)
Porcine	MDLWQLLLTL	AVAGSSDAFS	GSEATAAILS	RAPWSLQSVN	PGLKT <u>N</u> SSKE	(33)
Human	MDLWQLLLTL	ALAGSSDAFS	GSEATPAVLV	RASQSLQRVH	PGL <u>E</u> TN <u>S</u> SGK	(107)
Sheep	MDLWQLLLTL	AVAGSSDAFS	GSEATPAFFV	RASQSLQILY	PVLETN <u>S</u> SGN	(2)
Bovine	MDLWQLLLTL	AVAGSSDAFS	GSEATPAFLV	RASQSLQILY	PVLETN <u>S</u> SGN	(86)

Amino acids 1-17 are signal peptide. The thrombin cleavage sites are indicated by bold letters. The potential Asn-linked glycosylation sites (NXS/T, X can be any amino acid except Pro and Asp) are underlined.

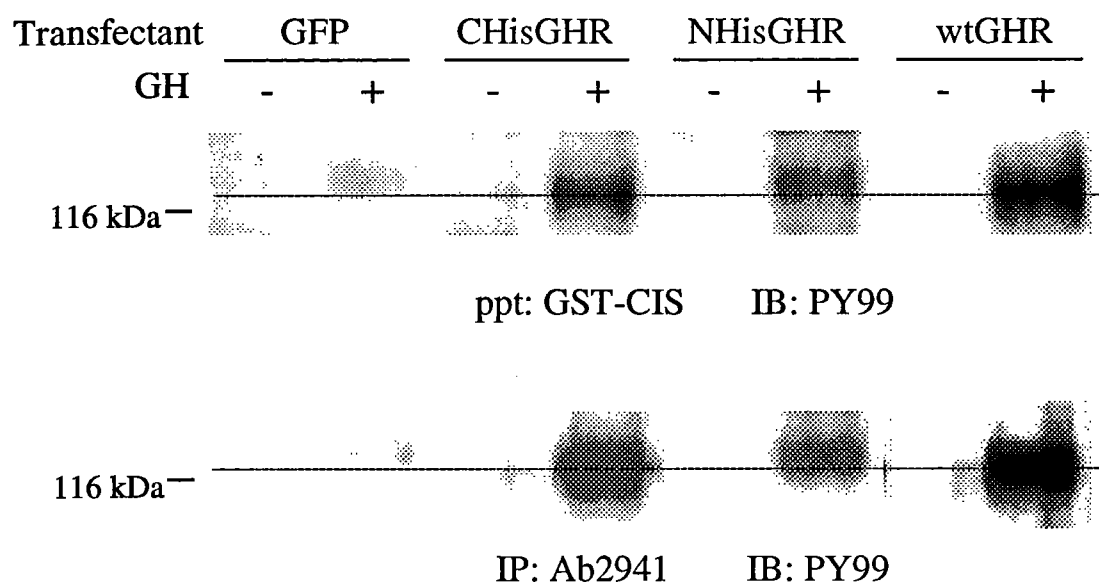
Regardless of its significance, the identical eletrophoretic mobility shifts of both the GHR and p120 after thrombin digestion indicated that the two proteins shared another characteristic: a thrombin cleavage site that produced the same reduction in their apparent molecular weights.

### *The p120 protein in cells expressing His-tagged GHR*

GHR tagged with 6×histidines at either the C-terminus (CHisGHR) or the N-terminus (NHisGHR) were expressed in HEK 293 cells. CHisGHR retained the typical electrophoretic mobility of wtGHR (120 kDa) as detected by PY99, but for some unknown reason, addition of the His-tag on the N-terminus of GHR increased its apparent mass to about 130 kDa on SDS-PAGE. This observation provided us with another opportunity to differentiate p120 from the GHR. Thus, plasmids containing cDNA for wtGHR, CHisGHR or NHisGHR were transiently transfected into HEK 293 cells. The cells were lysed after GH stimulation and precipitated by either Ab2941 or GST-CIS. The precipitates were analyzed by immunoblotting with  $\alpha$ PY. As shown in Figure 20, the mobility of the NHisGHR was retarded compared to that of wtGHR and CHisGHR. Examination of the tyrosine phosphorylated protein brought down by GST-CIS from cells expressing NHisGHR revealed that it, too, was retarded on SDS-PAGE to a similar extent, while its counterpart in cells expressing CHisGHR remained at 120 kDa. Retardation of the electrophoretic mobilities for both the GHR and p120 in cells overexpressing the NHisGHR implied that either addition of the His-tag to the N-terminus of the GHR conferred a similar anomalous increase in size to p120, or that p120 is the GHR.

### *Unsuccessful purification of p120 using GST-CIS pulldown and ProteinChip technology*

Although several lines of evidence described above suggest that the GHR is the major component of p120, the lack of direct evidence leaves the identity of p120 unclear.



**Figure 20.** Expression of His-tagged GHRs in HEK 293 cells. Plasmids containing cDNA for wild type rGHR (wtGHR), N-terminal His-tagged GHR (NHisGHR), C-terminal His-tagged GHR (CHisGHR) or green fluorescent protein (GFP) as control were transiently transfected into HEK 293 cells by Superfect reagent. 48 hours after transfection, the cells were treated with GH for 2 minutes and the lysates were immunoprecipitated with Ab2941 or GST-CIS. The precipitates were analyzed by immunoblotting with PY99. Lines were drawn to show the up-shift of the NHisGHR and the p120 in cells expressing NHisGHR. The results shown are representatives of two independent experiments.



The most straightforward way to identify p120 is to obtain amino acid sequence information from the purified protein. The identification approach involves digesting gel purified p120 with a site-specific protease (i.e. trypsin) and then mass analyzing the resultant peptides. The peptide masses can then be submitted to one of several mass database search algorithms that are available on web based servers. It may turn out that p120 is the GHR or a known protein whose protease digestion pattern can be found in the database. The available knowledge may help to understand p120's function in GH signaling or we can use an antibody (commercially available or produced by us) to further the investigation. If p120 is a novel protein, its partial sequences can be obtained by microsequencing, and degenerate oligonucleotide probes can be prepared for its cloning.

GST fusion proteins have been successfully used to affinity purify tyrosine phosphorylated proteins in at least two studies (135, 136). The efficacy of the pulldown experiment using GST-CIS fusion protein facilitated purification of p120. The thrombin cleavage site between GST and CIS sequences in the GST-CIS fusion protein would provide a specific criterion to selectively elute only CIS and CIS-binding protein(s). However, CIS was not released from GST portion of the fusion protein after thrombin digestion, possibly by some tertiary structure involvement with GST. Therefore, we sought a two-step purification procedure for p120.

The minimum amount of protein required for mass spectrometric analysis is 5 pmole, that is 600 ng of p120. Assuming there are 1000 copies of p120 in a single 293A cell,  $3 \times 10^9$  cells will be needed to purify sufficient protein on a yield of 10%. At least

1000:1 for the ratio of GST-CIS to p120 was used to allow efficient capture of the protein. GST-CIS fusion protein was prepared from bacterial culture and bound to a glutathione Sepharose 4B column. Cell lysates were prepared from  $3 \times 10^9$  GH-stimulated 293A cells grown in 160 culture dishes (145 mm $\times$ 20 mm). The lysates were first passed through the prebound GST glutathione Sepharose 4B column to reduce nonspecific binding, and then through the prebound GST-CIS glutathione Sepharose 4B column. After extensive washing, the fusion protein and proteins associating with it were eluted with reduced glutathione. The eluates were concentrated and immunoprecipitated with PY99-agarose for a second purification step. The final products were analyzed by SDS-PAGE and visualized by silver staining of the gel. Although p120 had been greatly enriched on the GST-CIS column, eluates contained significant amounts of contaminating proteins that produced a lot of interference to the identification to the p120 band on the silver stained gel. Further purification by anti-phosphotyrosine PY99-agarose turned out to be of low efficiency. Because our ability of detecting and purifying p120 relies on its tyrosine phosphorylation status, the low efficiency could be caused by the dephosphorylation of p120 with time. Several attempts at purification have been fruitless.

We also tried to take advantage of the ProteinChip SELDI (surface enhanced laser desorption/ionization) technology, which might have provided us an alternative way to characterize p120 at femtomole amount and more rapidly. It uses ProteinChip Arrays containing chemically or biochemically treated surfaces for specific interaction with proteins of interest. The mass profile of the proteins bound to each of the proteinchip array surfaces is quantitatively detected in minutes by the ProteinChip Reader. Proteases

can be used to produce a peptide map of a purified protein bound to the chip by on-chip digestion.

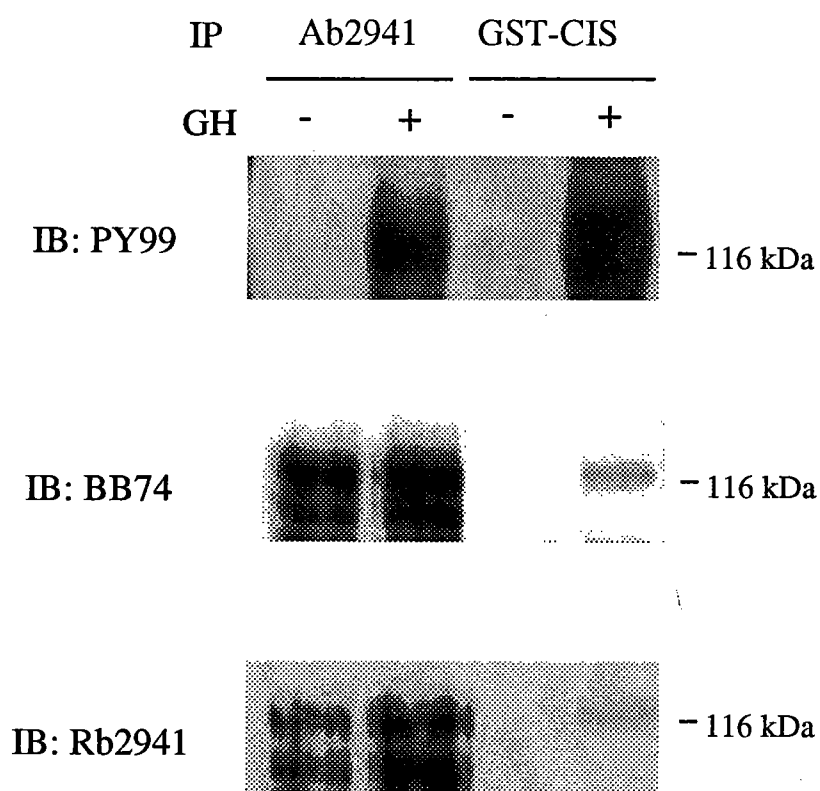
Based on the tyrosine phosphorylation status of GST-CIS or  $\alpha$ CIS precipitated p120, two different chips were selected: immobilized metal affinity capture chip IMAC3 and preactivated surface chip PS1. IMAC3 can be coated with  $\text{Fe}^{3+}$ , which can bind the phosphate groups on tyrosine phosphorylated p120. Alternatively, PS1 can be coated with  $\alpha$ PY which recognizes p120 as well. Ideally, IMAC3 would be the first choice because of its low "noise" after on-chip protease digestion. We set up a collaboration with Dr. S-M Ho of the Surgery Department who owns the system. However, even with the help from the manufacturer, Ciphergen Biosystems, Inc., numerous preliminary experiments were unsuccessful, and we abandoned this method when progress along other lines made this approach less critical.

*p120 is recognized by GHR antibodies in scale-up experiments*

The strongest evidence against the hypothesis of p120 being the GHR is that GHR antibodies failed to recognize it despite the strong signal detected by  $\alpha$ PY. Because there are 8-10 tyrosines in the GHR intracellular domain, it is possible that the GHR has multiple tyrosines phosphorylated, and thus can bind multiple copies of  $\alpha$ PY to give a much stronger signal than a single tyrosine phosphorylated protein with the same amount of protein as detected by  $\alpha$ PY. The mass abundance of this multiple phosphorylated protein might be just below the detection threshold of GHR antibodies. The low protein abundance on the membrane could be the reason why the GHR antibodies could not

recognize p120, assuming p120 was the GHR. Therefore, it was necessary to test if the GHR antibodies could recognize p120 when there is sufficient mass of p120 on the membrane. Though the purification of p120 by GST-CIS was unsuccessful, we were able to enrich p120 on the GST-CIS beads from the lysate of  $10^7$  GH-stimulated 293A cells, which represents 10 times more sample than used in the regular immunoprecipitation or pulldown experiments. The precipitates were analyzed by immunoblotting with GHR antibodies again. Interestingly, in addition to the GHR bands detected in Ab2941 precipitates, this time both Ab2941 and BB74 detected a faint band at the position of 120 kDa from GST-CIS precipitates, but only in samples from GH-stimulated cells (Figure 21). Therefore, at least some of the p120 signal comes from the GHR. This result provides direct evidence supporting p120 being the GHR.

Western blot analysis of GHR using either Ab2941 or BB74 indicates 2 distinct bands, one corresponding to 100 kDa and one corresponding to ~120 kDa. Similar findings have been obtained by others (172). GH-dependent tyrosine phosphorylation is evident only in the 120 kDa band. Of the two bands of the GHR, 120 kDa and 100 kDa, as detected by both Ab2941 and BB74, only the tyrosine phosphorylated 120 kDa form co-precipitates with GST-CIS in amounts detectable by Ab2941, implying that tyrosine phosphorylation is a prerequisite for the association of CIS with the GHR. It may be recalled that we failed to detect GHR in the pulldown experiment from adipocytes even using  $4 \times 10^7$  cells. This is not surprising considering the difference of level of GHR expressed in adipocytes (6,000-12,000 copies/cell) and 293A cells (120,000-240,000 copies/cell). Furthermore, considering the difference of GHR protein abundance from



**Figure 21.** p120 is recognized by GHR antibodies in scale-up experiments. Lysates of  $7 \times 10^7$  293A cells stimulated with GH were precipitated with Ab2941 or GST-CIS. The volume of lysates used for GST-CIS pulldown were 4 times as big as those used for Ab2941 precipitation. The precipitates were loaded on 3 gels for SDS-PAGE followed by immunoblotting with PY99, BB74 or Ab2941. The results shown are representative of three independent experiments.

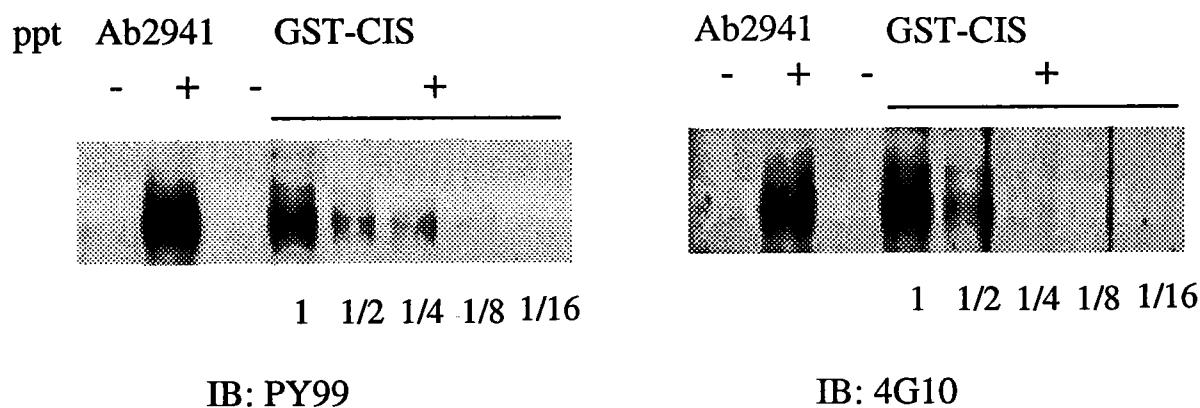
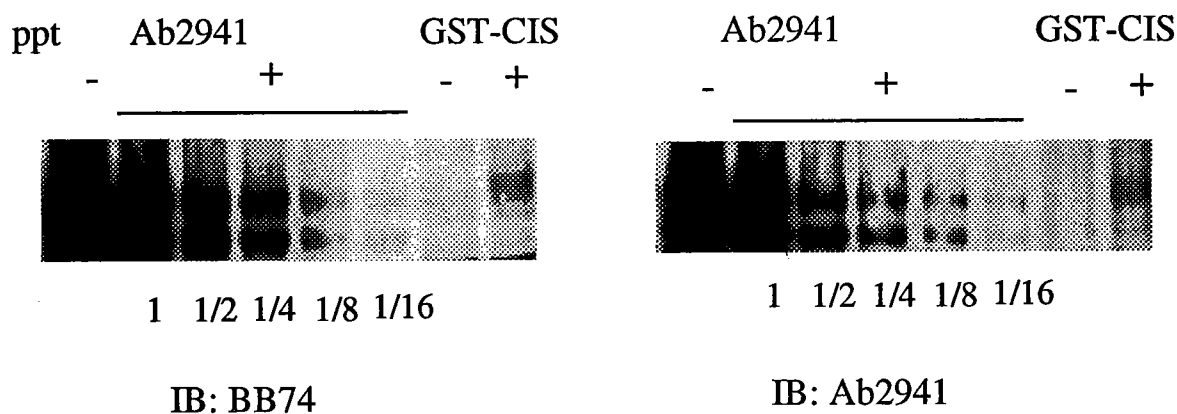
both precipitates as detected by both GHR antibodies, the GHR precipitated by GST-CIS showed a higher density of tyrosine phosphorylation than the GHR precipitated by Ab2941 as detected by  $\alpha$ PYs, assuming the similar efficiency for Ab2941 immunoprecipitation and GST-CIS pulldown. Assuming all of the p120 signal comes from the GHR, there are two explanations to this result. First, all the GHRs in cells may not be phosphorylated after GH stimulation. Second, there may be subtypes of phosphorylated GHR with different degrees of phosphorylation and different tyrosines phosphorylated in cells following GH stimulation. CIS may selectively bind to a subtype of GHR that is heavily phosphorylated or is phosphorylated on a particular tyrosine that is favorably detected by  $\alpha$ PY. On the other hand, it is also possible that the 120 kDa band is composed of the GHR and some other tyrosine phosphorylated protein(s) with the molecular mass at 120 kDa. If so, the other protein would have to be below detection level of  $\alpha$ PY because little residual band was detected at 120 kDa after GHR-T was cleaved by thrombin (Figure 19) or in cells overexpressing NHisGHR (Figure 20).

*Total cellular GHR, tyrosine phosphorylated GHR and CIS-associated GHR*

To further address this point, the GHR from both the GST-CIS and Ab2941 precipitates were analyzed for protein abundance and the extent of their tyrosine phosphorylation. Lysates from GH-stimulated 293A cells were precipitated by Ab2941 to obtain the total cellular GHR. Simultaneously, 5-fold larger samples of the same total lysates were subjected to pulldown by GST-CIS to obtain CIS-associated GHR. The precipitates from GST-CIS pulldown were serially diluted to match the tyrosine

phosphorylation density of GHR from Ab2941 precipitates, the samples were then loaded on two gels for SDS-PAGE arranged as shown in Figure 22A. After transfer, the membranes were immunoblotted with  $\alpha$ PYs. On the other hand, the GHR in Ab2941 precipitates was serially diluted to match the abundance of CIS-associated GHR. The samples were then loaded on two gels for SDS-PAGE arranged as shown in Figure 22B. After transfer, the membranes were immunoblotted with GHR antibodies. The immunoblotting analysis showed that the tyrosine phosphorylation signal of the undiluted CIS-associated GHR is close to that of GHR precipitated by Ab2941, as detected by two different  $\alpha$ PYs, PY99 and 4G10. However, the protein level of undiluted CIS-associated GHR is only between 1/4 to 1/8 of that of the GHR precipitated by Ab2941, as detected by two different  $\alpha$ GHRs, BB74 and Ab2941 (Figure 22B). Therefore, in order to obtain the same strength of signal from tyrosine phosphorylation, 4 to 8 times as much protein amount will be required for the GHR as for CIS-binding GHR. In other words, on the same protein level, CIS-binding GHR is 4 to 8 times as much tyrosine phosphorylated as the GHR. Either some tyrosine phosphorylated 120 kDa protein(s) other than the GHR also contribute to the tyrosine phosphorylation signal, or CIS interacts preferentially with multiply tyrosine phosphorylated GHR, or both.

An  $^{125}\text{I}$ -GH binding assay was employed to quantitatively estimate the amount of CIS that interacts with GHR. Adipocytes or 293A cells were stimulated with  $^{125}\text{I}$ -GH for 2 minutes. The cell lysates were then immunoprecipitated with Ab2941 or  $\alpha$ CIS in the presence of  $^{125}\text{I}$ -GH. Amounts of the GHR or CIS-associated GHR were calculated from

**A.****B.**

**Figure 22.** Comparison of tyrosine phosphorylation and protein abundance of CIS-associated GHR and total cellular GHR. Lysates from  $7 \times 10^7$  293A cells (stimulated with GH) were precipitated with Ab2941 or GST-CIS. Five fold larger aliquots of lysates were used for GST-CIS pull-down than for Ab2941 precipitation. After release from agarose beads, the immune complex precipitated from GH-stimulated cells was serially diluted with sample buffer from 1 to 1/16 and loaded on 4 gels for SDS-PAGE, arranged as shown above. After transfer to PVDF membrane, the membranes were immunoblotted with PY99 or 4G10 (A), or, BB74 or Ab2941 (B). The results shown are representative of two experiments.



cpm of the  $^{125}\text{I}$ -GH that co-precipitated with Ab2941 or  $\alpha\text{CIS}$ , respectively. As shown in Table 2, only 3.6% GHR in adipocytes and 0.50% GHR in 293A cells interact with CIS. These values are consistent with the results from Western blot analysis.

**Table 2.** Binding assay of GHR and CIS-associated GHR in adipocytes and 293A cells

	GHR (fmol/ $10^7$ cells)	CIS-associated GHR (fmol/ $10^7$ cells)	% CIS-associated GHR /GHR
Adipocytes	111	4.0	3.6
293A cells	1472	7.3	0.50

Data of adipocytes were provided by Dr. GP Frick.

The preclearance experiment described earlier (Figure 13A) showed that  $\alpha\text{CIS}$  could only co-precipitate no more than half of tyrosine phosphorylated GHR as judged from the tyrosine phosphorylation signal in immunoblotting, that is, CIS interacts with only a fraction of tyrosine phosphorylated GHR. Putting that observation together with results shown in Figure 22, it suggests that either only a small percentage of total cellular GHR is tyrosine phosphorylated after GH stimulation and CIS interacts with a small fraction of tyrosine phosphorylated GHR, or that CIS selectively associates with multiply tyrosine phosphorylated GHR. Both alternatives are valid possibilities considering that there are 10 tyrosines in the intracellular domain of the rat GHR.

## Discussion

The direct evidence supporting the p120 protein being the GHR is that two GHR antibodies can recognize p120 in scale-up experiments. In addition we have demonstrated that p120 and the GHR share several characteristics, including:

1. Tyrosine phosphorylation upon GH stimulation;
2. The same molecular weights, 120 kDa;
3. Interaction with CIS;
4. N-glycosylation to similar extent as judged by similar electrophoretic mobility shifts after Endo F digestion;
5. The similar electrophoretic mobility shifts after thrombin digestion;
6. Retarded electrophoretic mobility in cells overexpressing NHisGHR.

On the whole these data make it clear that the GHR is at least one major component in the p120 band. We cannot rule out, at this point, the possibility that there might be other tyrosine phosphorylated 120 kDa protein(s) that interact with CIS and that contribute to the p120 signal. But such protein(s), if there are any, have to be of sufficiently low abundance to be undetectable in any of the electrophoretic mobility shift studies described.

The detection of the GHR in GST-CIS precipitates prepared from 293A cell lysates demonstrated that CIS interacts with the GHR. Although the low abundance of the GHR in adipocytes prevented us from obtaining a similar result, apparently the same interaction exists in adipocytes as CIS co-precipitates with GHR antibody (Figure 13B). The demonstration that CIS interacts with the GHR in both adipocytes and cultured cells showed, for the first time in pure mammalian cell systems, that CIS can interact with

another member of the cytokine receptor superfamily, in addition to EpoR, IL-2R and IL-3R. Thus it is likely that CIS exerts its effects on cytokine signaling through direct interaction with receptors of cytokines. Our results not only are consistent with later findings showing that CIS binds to GST fused GHR intracellular domain (142) (83), but further demonstrate that CIS is capable of binding to the full length GHR naturally expressed in primary adipocytes as well as to GHR overexpressed in cultured cells.

The 4-8 times difference of the tyrosine phosphorylation signal between the GHR and the CIS-binding GHR explains the failure of GHR antibodies to detect p120 in regular immunoprecipitation/pulldown and immunoblotting experiments. Even though there may be other CIS-binding 120 kDa proteins that contribute to the p120 phosphotyrosine signal, it appears that the CIS-associated GHR represents the majority of it. Since the intracellular domain of the rat GHR has 10 potential tyrosine phosphorylation sites, it is possible that CIS selectively bind to multiply tyrosine phosphorylated GHR. This suggests that GHR molecules are not uniformly tyrosine phosphorylated in GH-stimulated cells. The exact tyrosine phosphorylation patterns of GHR are unknown. Mutational analysis showed that phosphorylation of Tyr534 and Tyr566 in porcine GHR is required for GH-dependent activation of Spi2.1/CAT constructs (189), that of Tyr487 and Tyr534 is required for GH-promoted tyrosine phosphorylation of STAT5 (189) and that of Tyr333 and/or Tyr338 is required for GH-stimulated lipid and protein synthesis (112). Thus tyrosine phosphorylation patterns of the GHR may confer specificity to individual signaling pathways.

The identification of the interaction of CIS with the GHR is encouraging for further study of the mechanism of CIS action. Our data from immunoprecipitation and immunoblotting analysis of transfected cultured cells showed that the phosphorylated GHR bound to CIS represents only a small fraction of the total phosphorylated GHR present in both adipocytes (Figure 13A) and 293A cells (Figure 22). Using  $^{125}\text{I}$ -hGH binding to estimate the amount of the GHR that co-immunoprecipitates with CIS revealed that only about 3.6% GHR binds to CIS in adipocytes and that only about 0.50% GHR associated with CIS in 293A cells, which is consistent with results from Western blotting analysis. The difference between adipocytes and 293A cells may reflect the difference in abundance of the naturally produced GHR in adipocytes and the cDNA-derived GHR expressed in 293A cells. Overexpression of the GHR in 293A cells produced an abnormal stoichiometric relationship between GHR and endogenous CIS, but the significance of the association with so little of the GHR in primary adipocytes is puzzling. It is possible that only a small portion of the total cellular GHR is sufficient to transduce signals from extracellular ligand stimulation to an intracellular physiological response, or at least to transduce the signals to a CIS-related pathway. Studies on the relationship between binding and biological effects of human GH in rat adipocytes showed that different concentrations of ligand are needed to elicit various biological effects. Whereas the half-maximal concentration of GH needed to produce the response of refractoriness is as low as 5 ng/ml, 50-100 ng/ml of GH was required to produce half-maximal insulin-like effects: antilipolysis, glucose oxidation and leucine oxidation (78). This suggests different fractional occupancy of the GHR on the cell surface may confer

specificity leading to a specific biological response. On the other hand, co-immunoprecipitation experiments may have underestimated the amount of association between CIS and the GHR depending on how well the association is preserved during cell lysis and immunoprecipitation procedure, and on the relative efficiency of immunoprecipitating antibodies. Binding assay detects only the GHR that are bound by  $^{125}\text{I}$ -GH and thus it is likely to underestimate the amount of GHR that interacts with CIS if the three molecules are not simultaneously in the same complex. The actual fraction of the GHR that interacts with CIS may be bigger than 0.5-3%. Also, because the turnover of the GHR is rapid even in the absence of ligand, it is possible that a significant fraction of GHR found in the whole cell lysates is not accessible to GH in cells, but is in transit to or from the cell surface. Therefore, it is reasonable to assume that the binding of CIS to a fraction of the GHR in cells may lead to a specific CIS controlled pathway.

It is well known that the electrophoretic mobility of GHR is 114-140 kDa (depending on species) (107) (53) (56), which is significantly higher than its deduced molecular weight (~70 kDa). Removal of the N-glycosylation portion usually reduces the GHR to around 95 kDa. The amino terminal sequencing of GHR purified from rabbit liver showed that ubiquitin is associated with 20%-50% of the receptor molecule (107). Thus not all the GHR molecules are ubiquitinated and ubiquitination of the GHR is likely to occur after the molecule is internalized. The ubiquitination of proteins must occur on lysine residues because the  $\epsilon$ -amino group of this amino acid is essential for conjugation (89). There are several lysines in the N-termini of the GHRs that are conserved among species. Covalent binding of one or more ubiquitin moieties would add about 9 kDa per

ubiquitin molecule to the protein. Thus glycosylation and ubiquitination have been two factors known to contribute to the size difference of the GHR. However, other studies revealed that at any one time, only a few percent of rabbit GHR is ubiquitin-conjugated and the poly-ubiquitinated receptor appeared as a broad band, most of which is larger than 200 kDa, and ubiquitination is likely to be on the intracellular domain of the receptor (173). It is not clear if the same is true for the GHR from other species. Therefore, ubiquitination of the GHR is still an unsettled issue. On the other hand, if the Lys31 in the thrombin cleavage site in the rat GHR is one of the ubiquitination sites, it could explain the reduction of about 10 kDa (9 kDa from ubiquitin plus 1.7 kDa from the N-terminal fragment) from the receptor by thrombin digestion (from 120 kDa to 110 kDa)(Figure 19). Otherwise, there must be other factors covalently linked to the GHR, because there is no lysine upstream of the Lys31. The presence of a thrombin cleavage site near the N-termini of the GHR from some species but not others may provide a tool to discover such a factor. The retardation of the electrophoretic mobility of the N-terminal histidine-tagged GHR but not that of the C-terminal histidine-tagged GHR expressed in 293 cells (Figure 20) could be resulted from some small factor covalently bound to the N-terminus of the GHR that contributes disproportionately to its larger molecular mass. Such a factor might, like ubiquitin, be involved in regulation of the cellular fate of the GHR.

### Section III

#### Physiological Function Studies of CIS in GH Actions

The identification of the GHR as at least an important component of the CIS-binding p120 facilitated our study of the function of CIS in the GH signaling pathway. The ability of CIS to associate with the GHR upon GH stimulation implies that it might be capable of modulating GH signaling through its interaction with the GHR. As CIS/SOCS proteins are considered to be negative regulators of cytokine signaling, we questioned if CIS might negatively regulate GH activity. In this chapter, studies are focused on exploring the physiological functions of CIS in terms of GH signaling in both cultured cells and primary rat adipocytes.

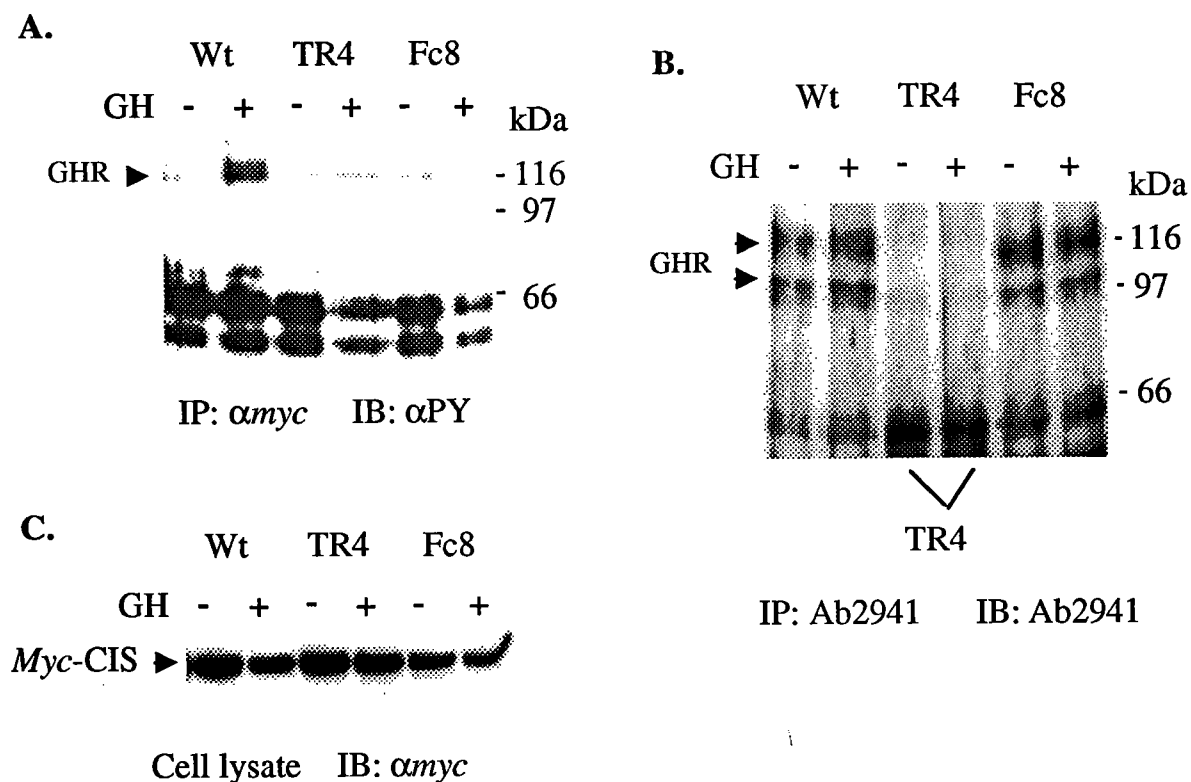
#### Results

##### *Identification of the site on GHR that interacts with CIS*

So far we have demonstrated that CIS interacts with tyrosine phosphorylated GHR following GH stimulation. However, the mechanism of interaction between CIS and the GHR was unclear. Previous data showed that only the tyrosine phosphorylated form of the GHR co-precipitates with GST-CIS (Figure 21) and CIS co-precipitates with GHR antibody only in GH-stimulated cells (Figure 13B), indicating the importance of phosphorylated tyrosines for CIS/GHR interaction. In the cytoplasmic domain of the GHR there are 8-10 tyrosines, depending on the species, that could be phosphorylated after GH stimulation. The importance of these phosphorylated tyrosines in GH action has

been emphasized in many studies (reviewed in (27) (88)). They provide the docking sites for signaling molecules with SH2 or SH3 domains. Considering the existence of an SH2 domain in CIS/SOCS proteins, it is possible that these tyrosines also serve as the interaction sites for CIS. To address this point, cDNAs for wild type porcine GHR (wt), a GHR truncation which lacks the carboxyl terminal half of its cytoplasmic domain (TR4) (amino acids 1-476)(195) and the GHR with all 8 tyrosines in the cytoplasmic domain mutated to phenylalanines (Fc8) (194) were transiently expressed in mouse L cells, together with cDNA for CIS. Co-immunoprecipitation of CIS with the GHR was then examined after incubating these cells with GH. The results showed that a 120 kDa band was detected by  $\alpha$ PY only in cells expressing wt GHR, but not in cells expressing TR4 or Fc8, although the wt GHR, TR4 and Fc8 were expressed at similar levels (Figure 23). The absence of tyrosine phosphorylated protein co-precipitating with CIS in Fc8 expressing cells suggests that either the GHR is the only tyrosine phosphorylated protein that interacts with CIS or that the binding of other tyrosine phosphorylated 120 kDa protein(s) requires tyrosine phosphorylation of the GHR. Furthermore, no tyrosine phosphorylated protein was observed that co-precipitates with CIS in TR4 expressing cells, indicating the carboxyl terminus of the GHR is required for CIS interaction with the GHR. Therefore, it is likely that CIS interacts with phosphorylated tyrosine(s) on the GHR, and these tyrosines are located near the carboxyl terminus of the GHR. This is consistent with later findings that CIS is capable of binding to GST-fused GHR intracellular domain fragment as short as 80 amino acids, provided the fusion protein is tyrosine phosphorylated (142).



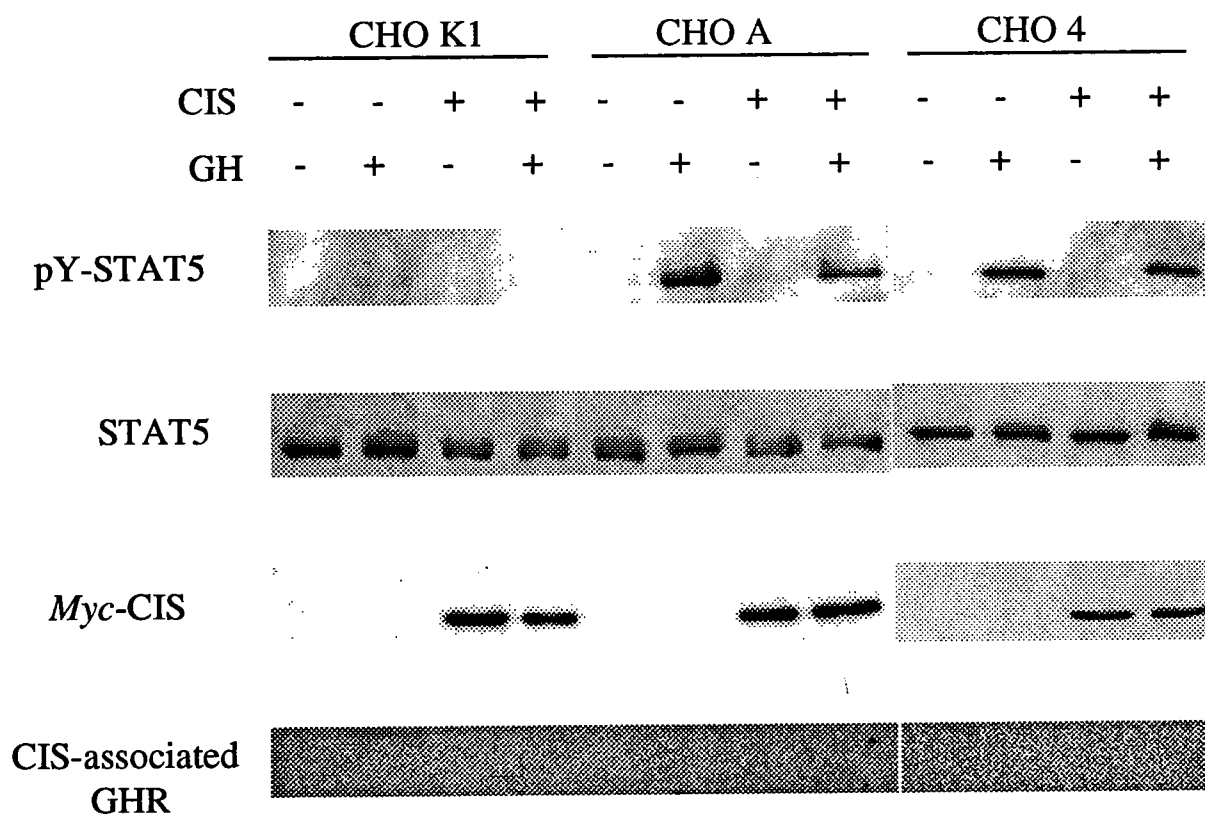


**Figure 23.** The tyrosines on the C terminus of GHR cytoplasmic domain are required for CIS/GHR interaction. Mouse L cells were transiently transfected with plasmid containing *myc*-tagged CIS cDNA together with plasmid containing cDNA of wild type porcine GHR (wt), GHR with the C-terminal half of cytoplasmic domain truncated (TR4) or GHR with all tyrosines in cytoplasmic domain mutated to phenylalanines (Fc8). The cells were incubated with or without hGH (500 ng/ml) for 2 minutes. **A.** The lysates were precipitated with  $\alpha myc$  and analysed by immunoblotting with  $\alpha PY$  to examine the interaction of GHR with CIS. **B.** The expression of GHRs was monitored by precipitating and immunoblotting With Ab2941. The molecular weight of Fc8 is about the same as that of wtGHR. TR4 has a predicted mobility corresponding to around 64 kDa, which routinely coincides with non-specific bands when immunoblotted with Ab2941. **C.** Cell lysates were analyzed by immunoblotting with HRP- $\alpha myc$  to check the expression of *myc*-CIS.

Five tyrosines in the C-terminal part of the porcine GHR were deleted in the TR4 construct, Tyr487, Tyr534, Tyr566, Tyr595 and Tyr627. Three of these tyrosines, Tyr534, Tyr566 and Tyr627 (equivalent to Tyr534, Tyr566 and Tyr626 in the rat GHR) have been identified as STAT5 binding sites in the GHR (84) (82). The lack of interaction between CIS and GHR in TR4 expressing cells suggests that those tyrosines may also be binding sites for CIS on the GHR. Competition of CIS for the STAT5 binding sites on the GHR may underlie at least some of the actions of CIS in GH signaling.

*CIS partially inhibits GH-induced STAT5 phosphorylation in CHO cells*

It has been shown that CIS partially suppresses Epo-induced STAT5 activation in HEK 293 cells and Ba/F3 cells. The CIS gene was proposed to be a direct target of STAT5 (120). In order to establish the relationship of CIS with GH-induced STAT5 activation, CIS cDNA was transiently expressed in CHO cells to examine the effect of CIS expression on GH-induced STAT5 phosphorylation. We found that when CIS was overexpressed in CHO cells that stably express the rat GHR, tyrosine phosphorylation of STAT5 is decreased compared to that in cells with no exogenous CIS (Figure 24). Furthermore, this phenomenon is only observed in CHO4 cells which express the full length rat GHR, but not in CHO4 cells which were stably transfected with cDNA that encodes the full length GHR, but express a functional GHR with a smaller molecular weight, 84 kDa (191). The molecular basis for the smaller molecular weight of GHR expressed in CHO4 cells is unknown. Our data suggest that the difference between the

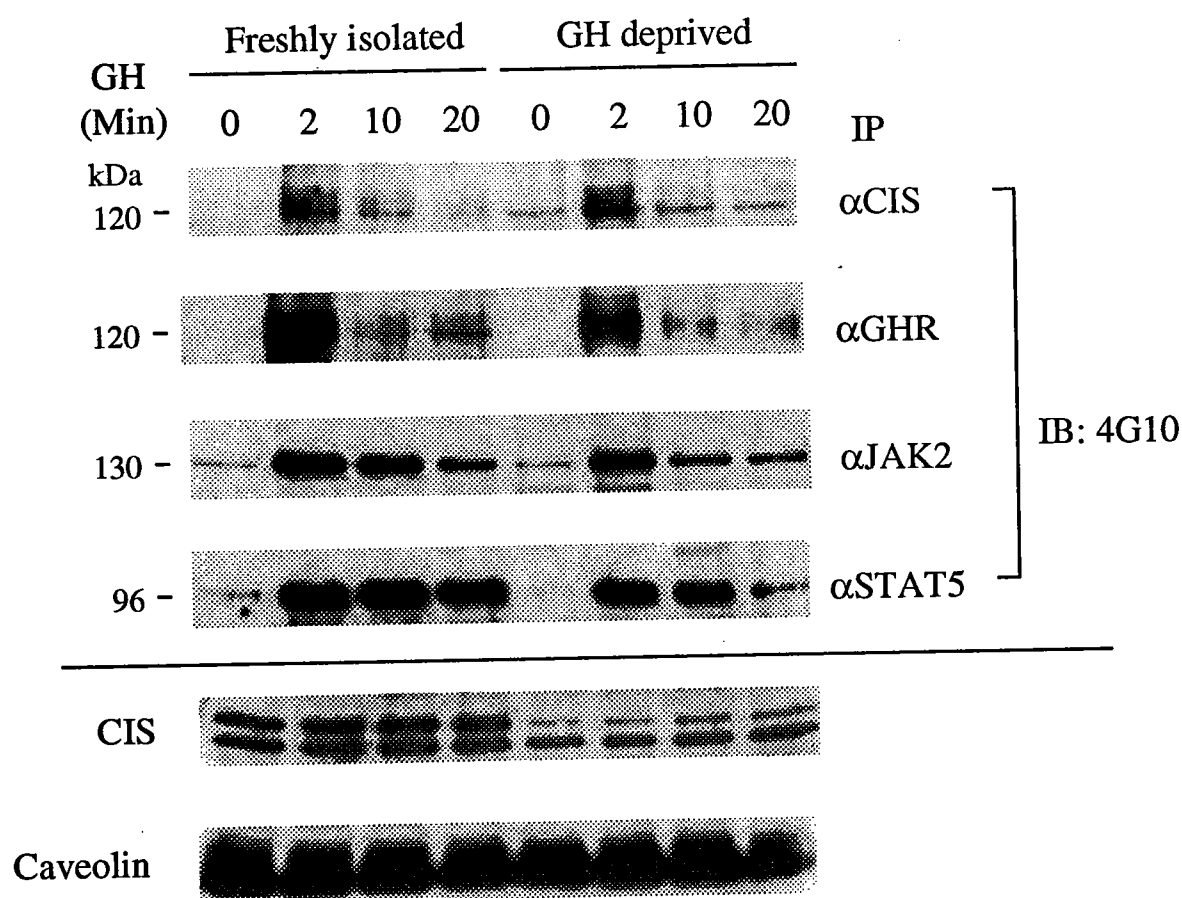


**Figure 24.** Overexpression of CIS in CHO cells partially inhibits GH-activated STAT5 phosphorylation. Parental CHOK1, CHOA and CHO4 cells were transiently transfected with pcDNA3/*myc*-CIS by Superfect transfection reagent. 48 hours after transfection, the cells were treated with or without 500 ng/ml hGH for 2 minutes. The lysates were incubated with anti-STAT5 and immunoprecipitates were collected on protein A coated agarose beads. The precipitates were resolved on SDS-PAGE followed by Western blotting with 4G10 to detect phospho-STAT5 (pY-STAT5). The membrane was then stripped and reprobed with anti-STAT5 to show the STAT5 abundance on each lane. The expression of CIS in cells was monitored by analyzing cell lysates with  $\alpha$ *myc*. The co-immunoprecipitation of CIS and the GHR was not detected by 4G10 in these cells (lower pannel). Similar results were obtained in another experiment of the same design.

120 kDa GHR in CHO A cells and the 84 kDa GHR in CHO 4 cells may account for the difference in CIS inhibition on GH-induced STAT5 activation. It is puzzling that the CIS/GHR interaction as shown by co-immunoprecipitation is barely detectable in CHO cells (Figure 24), although both CHO A cells and CHO 4 cells express more GHR than adipocytes (192) (125), implying that the CIS/GHR interaction is cell-type specific and CIS inhibition of GH-induced STAT5 activation may not rely on such interaction.

*CIS/GHR association and protein phosphorylation in refractory and sensitive adipocytes*

The finding of an inhibitory effect of CIS on STAT5 phosphorylation in cultured cells encouraged us to go back to adipocytes to investigate the effects of the CIS/GHR association and its correlation to the GH-induced insulin-like response. As the first step, the status of this association was examined in both freshly isolated (refractory) and GH-deprived (sensitive) adipocytes (Figure 25, upper panel). The co-precipitation of GHR with CIS was observed both in refractory adipocytes (freshly isolated adipocytes) and adipocytes that are sensitive to the insulin-like effects of GH. The appearance of CIS-associated GHR peaked at 2 minutes after GH stimulation, and rapidly declined to a low level by 20 minutes after GH stimulation in both refractory and sensitive cells. Attenuation of the co-precipitation of GHR with CIS shown here might be caused by reduction in the level of CIS protein or phosphorylated GHR, or by the dissociation of the two proteins. Examination of CIS protein levels from the same samples revealed that the reduction of CIS protein level does not account for the attenuation of CIS/GHR interaction. When comparing CIS abundance in freshly isolated cells and sensitive cells,

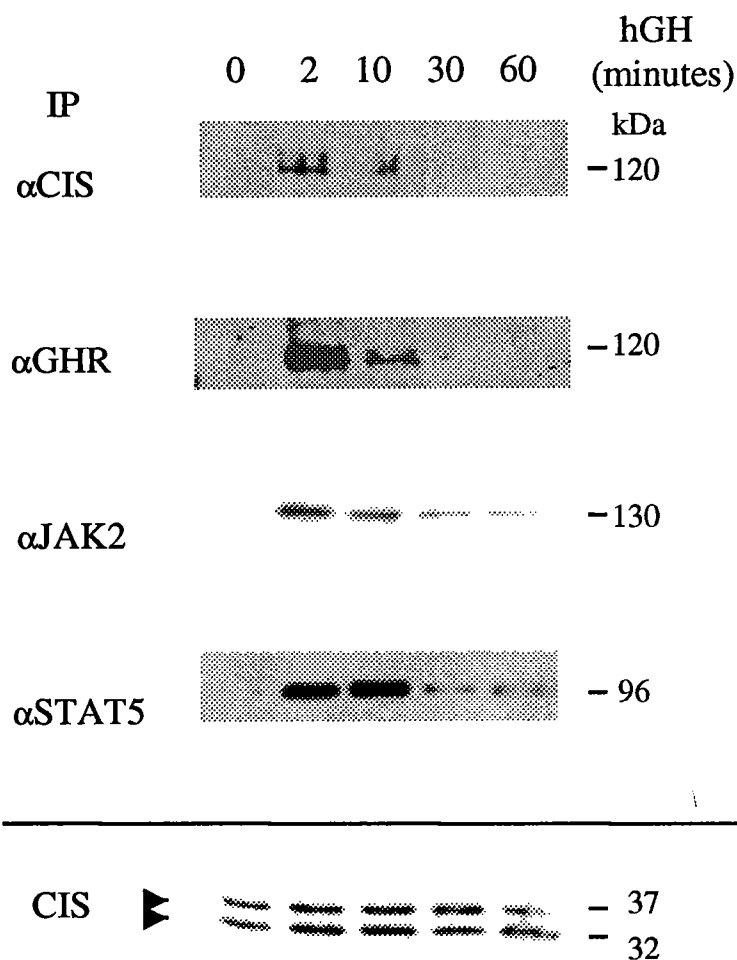


**Figure 25.** CIS and tyrosine phosphorylation of key GH signaling molecules in adipocytes. Freshly isolated and sensitive adipocytes were stimulated with hGH for indicated lengths of time. The lysates were precipitated with antibodies against CIS, GHR, JAK2, STAT5. The precipitates were analyzed by immunoblotting with 4G10. CIS protein levels were monitored by blotting the membrane with  $\alpha$ CIS. Caveolin was detected directly from lysates to monitor the equality of sample size. Similar results were obtained from another experiment of the same design.

apparently the three-hour deprivation of GH greatly reduced the CIS level, especially the ubiquitinated 37 kDa form, suggesting that the reduction in the amount of CIS protein in cells is caused by both degradation and decreased synthesis. The reduction of CIS protein levels in sensitive cells further confirmed the dependence of CIS abundance on GH, which is consistent with what has been described in Section I. On the other hand, the appearance of the CIS-associated GHR shared a similar timecourse with the phosphorylated GHR. Therefore, it is most likely that the level of phosphorylated GHR determines the extent of CIS/GHR association. However, no significant difference in the CIS/GHR association was observed between freshly isolated and sensitive cells, nor was there a difference in the phosphorylation status of the GHR, JAK2 and STAT5 (Figure 25), indicating that these molecules are not likely to be key players in the termination of GH-induced insulin-like effects or the emergence of the refractoriness in adipocytes.

#### *Effects of CIS on GH/GHR signaling*

As described previously, CIS/SOCS proteins are thought to function primarily as negative regulators of cytokine signal transduction by down regulating the activity of key signal transducers, such as JAK2 and STAT5. Since CIS only associates with tyrosine phosphorylated GHR, such association may be capable of modulating the activities of GH signal transducers. We therefore chose the well characterized key molecules in GH signaling to evaluate possible effects of CIS by monitoring their phosphorylation status. These molecules include the GHR itself, JAK2 and STAT5 (Figure 26). Freshly isolated adipocytes were stimulated with GH for various lengths of time from 0 to 60 minutes.

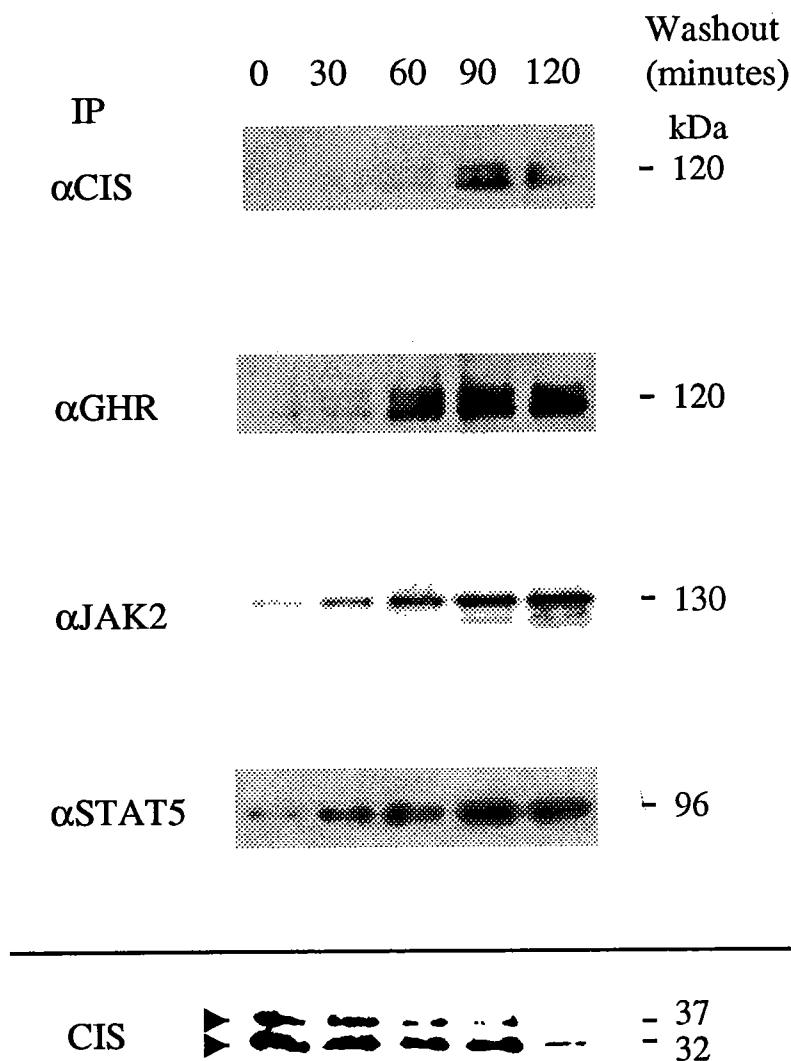


**Figure 26.** Effect of CIS on GH signaling. Freshly isolated rat adipocytes were treated with hGH (500 ng/ml) for the indicated times. Cell lysates were prepared and subjected to immunoprecipitation with the indicated antibodies. The samples were analyzed by SDS-PAGE followed by Western Blotting probed with 4G10. The bottom panel shows CIS protein levels at the corresponding time points as detected by  $\alpha$ CIS after immunoprecipitation with immobilized  $\alpha$ CIS. The results shown are representative of two experiments.

CIS-associated GHR, the GHR, JAK2 and STAT5 were precipitated from cell lysates with corresponding antisera and their phosphorylation status was visualized by Western blot analysis with 4G10 antibody. CIS protein levels were monitored by Western blotting using  $\alpha$ CIS. The results are shown in Figure 26. Phosphorylation of CIS-associated GHR, the GHR and JAK2 peaked at 2 minutes and that of STAT5 at 10 min after GH treatment. Phosphorylation of all three proteins decreased to very low levels 60 min after GH treatment. The amount of CIS protein did not show any dramatic change within the time period tested in this experiment. Thus, the amount of CIS does not appear to correlate with the phosphorylation status of all these GH signal transducers. The similar pattern of the phosphorylated GHR precipitated by Ab2941 and the CIS-associated GHR confirmed that it is the status of phosphorylated GHR that determines the degree of association between CIS and the GHR.

To further address the correlation of CIS/GHR association with the key molecules mentioned above, the responses of these molecules to a second exposure to GH was examined. Freshly isolated adipocytes were incubated in buffer with GH for one hour, followed by incubation in GH-free buffer for various lengths of time before they were challenged with GH again. The proteins were analyzed as described above. Data in Figure 27 showed that there was a period of latency in the response of all these proteins to re-phosphorylation after reintroduction of GH following 1 hour's GH treatment. Phosphorylation of the GHR, JAK2 and STAT5 remained low even 1 hour after the first exposure to GH, and recovery of the full response to GH was not seen until 90 minutes after the first exposure to GH. The CIS protein level declined gradually in the absence of





**Figure 27.** Desensitization of phosphorylated proteins stimulated by GH in rat adipocytes. Freshly isolated adipocytes were incubated in KRBG/1% BSA with 500 ng/ml hGH for 1 hour, then in GH-free buffer for the indicated washout times prior to re-stimulation of protein phosphorylation by addition of 500 ng/ml hGH for 2 minutes. Cell lysates were prepared and aliquots subjected to immunoprecipitation with the indicated antibodies. The samples were analyzed by SDS-PAGE followed by Western Blotting with 4G10. The lowest panel shows CIS protein levels at corresponding time points as detected by  $\alpha$ CIS after immunoprecipitation with immobilized  $\alpha$ CIS. The results shown are representative of two experiments.

GH, and the decline was more apparent for the ubiquitinated 37 kDa form. However, reduction of the protein level of the 37 kDa form of CIS did not interfere with the re-association of CIS with GHR when the GHR fully regained its responsiveness to GH 90 minutes after the initial GH challenge.

Results of the above two studies revealed no direct correlation of CIS protein level or CIS/GHR association with the changes in the GH-stimulated activities of GH signal mediators. No inhibitory effects of CIS could be discerned from these data, nor did CIS appear to be responsible for initiation or termination of GH-induced phosphorylation of GHR, JAK2 and STAT5 in adipocytes.

## **Discussion**

To date except for the tyrosine phosphorylation of SOCS3 that occurs in IL-2 stimulated cells (36), no basal or GH-induced tyrosine phosphorylation of CIS/SOCS proteins has been reported. The relatively constant abundance of CIS in freshly isolated or sensitive adipocytes does not appear to account for the rapid and dramatic changes of the tyrosine phosphorylation status of the GH signal transducers, namely, the GHR, JAK2 and STAT5. Overexpression of CIS in CHO cells resulted in partial inhibition of GH-induced STAT5 phosphorylation, but endogenously expressed CIS in adipocytes did not appear to be responsible for the changes of STAT5 phosphorylation following GH stimulation, washout or rechallenge. The failure to implicate CIS or the tyrosine phosphorylation status of the GHR, JAK2 and STAT5 as major players in refractoriness

to the insulin-like effects of GH in rat adipocytes suggests some other negative regulators might contribute to this phenomenon.

Our results do not rule out the possibility that CIS exerts its effects not through its protein abundance in cells, but through its availability. CIS could be bound by another protein at rest, and be released when the cells were activated (GH stimulated). The free CIS would then be able to bind to the receptor and exert its effects. Indeed, the ability of several CIS/SOCS proteins to bind to GST-fused GHR intracellular domain and produce dramatic different inhibitory effects on GH signaling (83) suggests that binding to the receptor is not sufficient to account for inhibition of GH signaling. In refractory and sensitive cells association of CIS with the GHR followed the same timecourse as that of the GHR tyrosine phosphorylation. Thus, if CIS has any function in refractoriness, it must act through other mechanism(s) in addition to its interaction with the GHR. The protein that traps CIS might be tyrosine phosphorylated and have a molecular mass of 120 kDa. The existence of another 120 kDa CIS-binding protein might explain the heavy tyrosine phosphorylation signal from the GST-CIS precipitates compared to the weak signal from the same amount of the same precipitates but detected by the GHR antibodies (Figure 22). The lack of the tyrosine phosphorylated protein that co-precipitates with CIS in Fc8 expressing mouse L cells suggests if there exists such a CIS-binding protein, its interaction with CIS is dependent on the tyrosine phosphorylation of the GHR.

An ideal way to study the effect of CIS in refractoriness would be to overexpress or knockout CIS in adipocytes and then determine if any changes of the insulin-like responses to GH are noted in these cells. CIS transgenic mice have been produced (121),

but except for growth retardation and scarcity of adipose tissue, no changes of GH-induced activities in their adipocytes have been reported. Because adipocytes are terminally differentiated cells, they cannot be transfected like dividing cells. Adenovirus carrying CIS cDNA has been generated and used to transduce adipocytes. However, it takes at least 48 hours for protein expression before any measurement can be made. Adipocytes can be cultured, but conditions for producing refractoriness under these circumstances are not known. In our experience, adipocytes lose a considerable amount of fat after 3 hours incubation at 37°C in KRPG buffer containing 5.5 mM glucose and 10 mg/ml BSA. Forty-eight-hour incubations of adipocytes in DMEM not only results in a major loss of cells, but also changes cell morphology. Besides, we were unable to determine if the incubated adipocytes still retain the physiological characteristics of freshly isolated adipocytes. Differentiated primary preadipocytes obtained from rat adipose tissue or 3T3-F442A cells did not appear to be good substitutes either on this aspect. Therefore, so far, the primary adipocyte is the only cell model that has been used to investigate refractoriness. Nevertheless, the finding of an alternative cell system that can be genetically engineered would definitely advance further study.

The factors that cause delayed recovery of the response of the GHR, JAK2 and STAT5 to GH-stimulation after initial exposure to GH are not known. Full responsiveness to GH-stimulation was regained in about 90 minutes after the removal of GH, a time that differs from the 3 hours required for termination of refractoriness after excision of adipose tissue. However, since tyrosine phosphorylation is usually an early response to cytokine stimulation, responsiveness of unknown molecules downstream of

the pathway may be more reflective of refractoriness. The factors that are responsible for the appearance of refractoriness are likely to be further downstream of or distinct from JAK-STAT pathway. Furthermore, negative regulatory proteins induced by GH through the JAK-STAT or other pathways require time for synthesis and to be cleared by degradation. Interestingly, the temporally related effects of GH on STAT5 activity in liver cell lines reported as measured by electrophoresis migration shift assay (EMSA) or activation of a reporter gene are remarkably similar to our findings in adipose tissue (55). In the liver, these effects are of particular importance for the sexually dimorphic regulation of hepatic genes that depend upon intermittent stimulation with GH as seen in male rats as compared to the nearly constant stimulation by GH seen in female rats. Upon initial stimulation with GH, STAT5 activity increased to a maximum at around 1 hour and returned to baseline within 1 hour after removal of GH (64). A GH-free interval of at least 2.5 hours was needed before GH could again fully activate STAT5, reminiscent of the period of refractoriness in adipocytes. Cycloheximide added along with GH maintained STAT5 activity for at least 4 hours suggesting, as with termination of the insulin-like response (69), that a short-lived protein is somehow involved in turning off the GH response (65) (55). Inhibition of phospholipase C and blockade of serine/threonine kinases also prolonged the activated state of JAK/STAT signaling (55). Although the role of changes in  $[Ca^{2+}]_i$  have not been explored in this light in hepatocytes, it was noteworthy that inhibition of phospholipase C and protein kinase C in adipocytes prevents GH-induced increase in  $[Ca^{2+}]_i$ , and that elevated  $[Ca^{2+}]_i$  is associated with refractoriness in adipocytes. No short-lived protein has been identified that

contributes to the delayed response of STAT5 to GH stimulation in liver cells. Induction of CIS by GH was hypothesized to be one mechanism by which sexually dimorphic GH signaling via STAT5b is achieved in the rat liver. Although GH regulates CIS mRNA expression in rat liver and in cultures of primary rat hepatocytes, and constitutive expression of CIS inhibited the GH-induced transactivation of a STAT5-responsive reporter gene construct (100), there is no direct evidence to confirm that hypothesis yet. As neither the mechanisms underlying desensitization of GH-stimulated STAT5 activity in liver cells nor refractoriness to insulin-like responses of GH in adipocytes are clear at present, it is hard to predict how much these two phenomena are related. We suspect these are separate events, since so far no evidence has implicated STAT5 in refractoriness.

The lack of difference of the GH-induced phosphorylation of the GHR, JAK2 and STAT5 between refractory and sensitive adipocytes suggests that the tyrosine phosphorylation status of these mediators may not be the determinants of refractoriness or that refractoriness results from a more distal change. However, the data described here on the tyrosine phosphorylation of proteins differs from those reported by Eriksson et al (53). In their study, no tyrosine phosphorylation of GHR and JAK2 was observed in freshly isolated and hence refractory adipocytes after 10 minutes of GH stimulation. Their data imply activation of phosphotyrosine phosphatase or suppression of tyrosine kinase activities in refractory cells. No confirmation of these results has been published by others researchers. Furthermore, because the refractory state is the one that prevails *in vivo* it is unlikely that JAK2 cannot be activated by GH *in vivo* in adipocytes. Clearly

JAK2 is activated in liver, muscle (31) and other tissues (181) at times of refractoriness to insulin-like stimuli. Also, although the lipolytic action of GH appears to involve the STAT5 proteins, an insulin-like effect of GH on glucose conversion to lactate was observed in adipose tissue from mice deficient in STAT5a/b, suggesting the insulin-like effects of GH on glucose metabolism involve different mechanisms than GH-stimulated STAT5 activation (54). Therefore, it is likely that JAK2/STAT5 pathway remains active in refractory cells.

The requirement of phosphorylated tyrosines located on the C-terminal part of the GHR for interaction with CIS suggests that CIS may interact with the GHR through its SH2 domain. Although a functional SH2 domain of CIS was not required for its interaction with IL-2R  $\beta$  subunit in transfected HEK 293T cells, phosphotyrosine binding nevertheless was essential for expression of inhibitory action of CIS (9). The SH2 domain of SOCS1 was also found to be required for its binding to JAK2 (133), though the isolated SH2 domain of SOCS1 failed to inhibit JAK2 autophosphorylation in HEK 293 cells and had no effect on the transcriptional activity of the *c-fos* promoter in Ba/F3 cells (52). In the case of SOCS3, an additional 46 amino acids C-terminal to the SH2 domain seem to be needed for SOCS3 binding to the GST-fused GHR intracellular domain, as well as inhibition of GH-induced signaling (83). Therefore, it is possible that the SH2 domain needs a particular tertiary structure to play its role, or that the simultaneous binding of an SH2 domain and another motif in the CIS/SOCS is required. It has been predicted recently that two functional domains are required, one, which includes the SH2 domain, is involved in binding to phosphotyrosines in cytokine

receptors or JAK kinases, and the other, which includes the N-terminal domain, is involved in the actual inhibitory effects (83). Also, individual members of the family may act differently, as the N-termini of each of the proteins share little homology, which may confer specificity to individual action.

Although the mechanism of action of CIS is unclear, currently several possibilities have been postulated (204) (8): 1) CIS could be an adaptor protein that interacts with other proteins through its SH2 domain, N-, or C- terminal domain; 2) It could directly block tyrosine phosphorylated motifs on receptors, preventing their coupling to stimulating signal molecules; 3) Because of the short half-life of CIS, it may act as a scavenger of phosphorylated proteins, targeting them for degradation.

The relationship of CIS to STAT5 has not been well defined to date. There are four MGF boxes (TTCNNNGAA, consensus STAT5 recognition sequence) in the promoter region of the CIS gene, and disruption of the MGF boxes abolished the Epo-dependent activation of CIS promoter (120), suggesting that the CIS gene is a direct target of STAT5. Overexpression of CIS partially inhibits Epo-induced STAT5 phosphorylation (120), as well as IL-2-mediated activation of STAT5 and Lck-mediated phosphorylation of the IL-2R  $\beta$  subunit in 293 cells (9). Depending on the reporting systems used to examine the effect, overexpression of CIS in cultured cells showed partial or no inhibitory effects on GH-induced STAT5 activation (Table 3). For example, CIS did not show any effect on GH-stimulated transcription of the Spi2.1 promoter which is STAT5-dependent (83). In the present study, we have shown that overexpression of CIS in CHO cells partially inhibited GH-induced STAT5 phosphorylation, as it did to



**Table 3.** Effects of CIS on GH-induced STAT5 activity

Reporting System	Effects	Reference
EMSA using an STAT5 response element derived from rat $\beta$ casein gene	Partial inhibition	(142)
Rat ntcp/luciferase reporter gene activity (4 copies of a STAT5 response element)	Almost complete inhibition	(142)
Spi2.1 promoter/CAT activity	No inhibition	(83) (3)
EMSA using a STAT5 binding element from Spi2.1 promoter	Partial inhibition	(83)
Immunoprecipitation and Western blotting for STAT5 phosphorylation in CHO cells	Partial inhibition	This study

EMSA: electrophoretic mobility shift assay

Ntcp: hepatic Na<sup>+</sup>/taurocholate cotransporting polypeptide (61)

CAT: chloramphenicol acetyltransferase

Epo-induced phosphorylation of STAT5 in hematopoietic cells that express CIS and STAT5. However, we were unable to correlate the change of CIS abundance with GH-induced phosphorylation of STAT5 or other key GH signal transducers in adipocytes. The lack of inhibition by CIS on STAT5 phosphorylation in adipocytes might imply that CIS has different roles in mitogenically active cells and terminally differentiated adipocytes. Alternatively, it is possible that CIS can inhibit STAT5 phosphorylation only when overexpressed in great excess but not when present in physiological concentrations. Inefficient expression (or protein instability) of CIS in transfected cells has also been suspected to account for the lack of inhibition in some studies (142). In addition, we suspect the interaction of CIS/GHR and/or the inhibitory effect of CIS on GH-induced STAT5 activity is cell type-dependent and may involve other factors.

The results observed in the present study are consistent with recent reports by Ram et al. (142), in which CIS/SOCS proteins were divided into three categories according to their inhibition of GH-stimulated STAT5 signaling. SOCS1 and SOCS3 were highly potent inhibitors, CIS and SOCS2 were somewhat weaker inhibitors when expressed at similar protein level, and SOCS6 was non-inhibitory. In that report it was proposed that CIS/SOCS proteins inhibit GH-stimulated STAT5 signaling by three distinct mechanisms. SOCS1 acts at the level of JAK2 tyrosine kinase, SOCS3 acts at the level of the membrane-proximal tyrosine residues Tyr333 and Tyr338 of the GHR, and CIS and SOCS2 act on the level of membrane-distal tyrosine residue(s). The demonstration of individual proteins acting on distinct molecular targets of the GHR/JAK2 complex suggests that each of the GH-inducible CIS/SOCS genes may play a unique role in termination of GH signaling or in selectively modulating GH signal pathways.

The specific consequences of CIS/SOCS binding to the receptor are not known at present. SOCS3 was found to bind to the SHP-2 binding site on gp130 and the activities of SOCS3 and SHP-2 were functionally linked and modulated (131) (154). As a phosphotyrosine phosphatase whose enzymatic activity can be increased by phosphorylation (189), SHP-2 could negatively regulate cytokine signaling as well. The linkage of SOCS3 with SHP-2 might determine the inhibitory specificity of particular downstream pathways. Although SOCS3 was also found to bind to the phosphotyrosines that are STAT5 binding sites in the GHR, SOCS3 did not appear to compete with STAT5 for binding to the GHR (83). In a proposed “bridge model”, SOCS3 would serve as an

inhibitory bridge by binding simultaneously to GHR phosphotyrosines 333/338 through its SH2 domain and to JAK2 via its kinase inhibitory sequence (residues 22-34) (142). However, CIS only produces incomplete inhibition of JAK2 activity, and mutation of tyrosines 333/338 in the GHR does not interfere with the inhibition of GH-induced STAT5 activity by CIS. Thus CIS does not seem to fit the bridge model. Instead, CIS may suppress STAT5 activity by binding to the membrane-distal phosphotyrosine residues in the GHR that are STAT5 binding sites. Because other upstream phosphotyrosine residues such as Tyr333 and Tyr338 can also support GH-stimulated STAT5 signaling, the multiplicity of the STAT5 binding sites in the GHR might explain the weak or non-inhibitory effect of CIS on GH-activated STAT5 signaling.

On the other hand, one implication of these studies might be that the inhibition of STAT5 activation is not the main aspect of the physiological function of CIS in cytokine signaling. One of the two forms of CIS, the 37 kDa form, was found to be ubiquitinated and degraded rapidly by proteosomes. Proteosome inhibitors appeared to protect the CIS-EpoR complex and thus inhibit the inactivation of the EpoR and STAT5 in human leukemic cells (188). SOCS1 was implicated in the hematopoietic-specific guanine nucleotide exchange factor VAV's ubiquitin-dependent degradation (42). It was also reported that the SOCS box mediates interactions with elongins B and C, which in turn may couple SOCS proteins and their substrates to the proteasomal protein degradation pathway (208). In this respect, it was proposed that CIS/SOCS proteins may act as adaptor molecules that target activated cell signaling proteins to the protein degradation

pathway. These findings suggest that targeting for protein degradation might be an efficient mechanism for CIS/SOCS protein to inhibit cytokine signaling.

## SUMMARY

Studies of the CIS/SOCS family suggest these proteins function as negative regulators of cytokine signaling. The present study focused on the investigation of the involvement of CIS/SOCS proteins in GH action, in particular, on the possible role of CIS/SOCS proteins in termination of GH-induced insulin-like effects and emergence of refractoriness in rat adipocytes. To answer the questions asked as the specific aims, the foregoing study found:

- I. GH up-regulates the mRNA expression of CIS, SOCS3 and SOCS1 genes in rat adipocytes.
- II. CIS associates with the tyrosine phosphorylated GHR upon GH stimulation of both adipocytes and cultured cells.
  - i. CIS binds to a small fraction of tyrosine phosphorylated GHR in both adipocytes and 293A cells.
  - ii. The GHR that CIS binds to is likely to be multiply tyrosine phosphorylated.
  - iii. CIS binds to the GHR through phosphotyrosines near the carboxyl terminus of its intracellular domain.
  - iv. There could be another protein (or proteins) that binds to CIS and has a molecular weight of 120 kDa, but if so, the abundance of this protein is below the detection threshold of anti-phosphotyrosine antibodies.

- v. A thrombin-cleavage site is found near the amino terminus of the GHR of rat, mouse and rabbit, but not of human, sheep and bovine.
- vi. No tyrosine phosphorylated proteins have been found to co-precipitate with SOCS3 in rat adipocytes.

III. Although CIS partially inhibited GH-induced STAT5 phosphorylation when overexpressed in CHO cells, no such effect was apparent in primary rat adipocytes. Neither the change of protein abundance of CIS nor its interaction with the GHR correlated with the time course of refractoriness. Therefore, CIS does not appear to be a major contributor to refractoriness.

## FUTURE DIRECTION

The future work will focus on two aspects: to investigate the physiological function of CIS in GH signal transduction and to discover molecules that regulate the insulin-like-effects of GH and cause refractoriness.

The strong and rapid binding of CIS to the tyrosine phosphorylated GHR in response to GH stimulation implies that this interaction is important. We would like to determine what CIS does by binding to the GHR. Since inhibition of GH-induced STAT5 activity does not appear to be the major function of CIS, the consequence of CIS/GHR interaction may lie downstream of STAT5 or lead to another pathway. Examination of end points of GH action in cells expressing a CIS mutant that does not bind to the GHR may lead to information on the significance of CIS/GHR interaction. Alternatively, the CIS gene can be knocked out from mice or tissue-specifically disrupted from adipose tissue to analyze the overall physiological function of CIS and its involvement in metabolism in adipose tissue. Also, the possible role of CIS in targeting the GHR for degradation has not been explored yet. We will examine the ubiquitination status of the GHR in the presence and absence of CIS in cultured cells to see if CIS potentiates GHR ubiquitination, as SOCS1 does to VAV (42). Proteasome inhibitors such as LLnL (N-acetyl-leucyl-nor-leucinal) and lactacystin (37) can be used to examine if CIS targets the GHR to proteosomal degradation, as it does to EpoR (188).

On the other hand, although CIS does not appear to regulate the insulin-like-effects of GH, results presented here and in other studies clearly demonstrate the

complexity of this physiological phenomenon. As our first attempt to get a broad range of understanding of gene regulation by GH, I have used differential display technology (108) to examine changes in the gene expression pattern in sensitive and refractory adipocytes. However, because of the limitation of the differential display technology that has also been demonstrated in other studies (24) (126), we could not confirm the differentiated gene expressions by Northern blot analysis. With the completion of the human genome project and the progress of genome projects of other species including mouse, a nearly complete platform is available for studying gene expression profiles. Instead of analyzing one or several genes a time, DNA microarray technology offers promise to analyze expression profiles of a great number of genes in altered physiological conditions, as exemplified in recent reports (35) (22) (6). This novel technology has also been extended to protein arrays, or "proteomics" (51). In these reports, altered gene profiles under disease condition were clearly demonstrated, and provided new directions in cancer classification and development of new drug targets. With a similar approach, i.e. by gene profiling the GH sensitive and refractory cells, the underlying mechanism which has been sought for decades could be elucidated. Furthermore developing an *in vivo* system that is capable of altering cell sensitivity to the insulin-like-effects of GH will greatly facilitate the study on this aspect of GH action.



## REFERENCES

1. Adams, T. E. (1995) Differential expression of growth hormone receptor messenger RNA from a second promoter. *Mol Cell Endocrinol* **108**:23-33.
2. Adams, T. E., L. Baker, R. J. Fiddes, and M. R. Brandon. (1990) The sheep growth hormone receptor: molecular cloning and ontogeny of mRNA expression in the liver. *Mol Cell Endocrinol* **73**:135-45.
3. Adams, T. E., J. A. Hansen, R. Starr, N. A. Nicola, D. J. Hilton, and N. Billestrup. (1998) Growth hormone preferentially induces the rapid, transient expression of SOCS-3, a novel inhibitor of cytokine receptor signaling. *J Biol Chem* **273**:1285-7.
4. Aguila, M. C. (1994) Growth hormone-releasing factor increases somatostatin release and mRNA levels in the rat periventricular nucleus via nitric oxide by activation of guanylate cyclase. *Proc Natl Acad Sci U S A* **91**:782-6.
5. Alexander, W. S., R. Starr, J. E. Fenner, C. L. Scott, E. Handman, N. S. Sprigg, J. E. Corbin, A. L. Cornish, R. Darwiche, C. M. Owczarek, T. W. Kay, N. A. Nicola, P. J. Hertzog, D. Metcalf, and D. J. Hilton. (1999) SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98**:597-608.
6. Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, L. M. Staudt, and et al. (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403**:503-11.
7. Altszuler, N. (1974) Actions of growth hormone on carbohydrate metabolism, p. 233-252, *Handbook of Physiology*, vol. IV. American Physiology Society, Washington.
8. Aman, M. J., and W. J. Leonard. (1997) Cytokine signaling: cytokine-inducible signaling inhibitors. *Curr Biol* **7**:R784-8.
9. Aman, M. J., T. S. Migone, A. Sasaki, D. P. Ascherman, M. Zhu, E. Soldaini, K. Imada, A. Miyajima, A. Yoshimura, and W. J. Leonard. (1999) CIS Associates with the Interleukin-2 Receptor beta Chain and Inhibits Interleukin-2-dependent Signaling. *J Biol Chem* **274**:30266-30272.

10. Anderson, N. G. (1992) Growth hormone activates mitogen-activated protein kinase and S6 kinase and promotes intracellular tyrosine phosphorylation in 3T3-F442A preadipocytes. *Biochem J* **284**:649-52.
11. Arden, K. C., J. M. Boutin, J. Djiane, P. A. Kelly, and W. K. Cavenee. (1990) The receptors for prolactin and growth hormone are localized in the same region of human chromosome 5. *Cytogenet Cell Genet* **53**:161-5.
12. Argetsinger, L. S., G. W. Hsu, M. G. Myers, Jr., N. Billestrup, M. F. White, and C. Carter-Su. (1995) Growth hormone, interferon-gamma, and leukemia inhibitory factor promoted tyrosyl phosphorylation of insulin receptor substrate-1. *J Biol Chem* **270**:14685-92.
13. Auernhammer, C. J., V. Chesnokova, C. Bousquet, and S. Melmed. (1998) Pituitary corticotroph SOCS-3: novel intracellular regulation of leukemia-inhibitory factor-mediated proopiomelanocortin gene expression and adrenocorticotropin secretion. *Mol Endocrinol* **12**:954-61.
14. Barton, D. E., B. E. Foellmer, W. I. Wood, and U. Francke. (1989) Chromosome mapping of the growth hormone receptor gene in man and mouse. *Cytogenet Cell Genet* **50**:137-41.
15. Baumann, G., H. B. Lowman, M. Mercado, and J. A. Wells. (1994) The stoichiometry of growth hormone-binding protein complexes in human plasma: comparison with cell surface receptors. *J Clin Endocrinol Metab* **78**:1113-8.
16. Baumbach, W. R., and B. Bingham. (1995) One class of growth hormone (GH) receptor and binding protein messenger ribonucleic acid in rat liver, GHR1, is sexually dimorphic and regulated by GH. *Endocrinology* **136**:749-60.
17. Baumbach, W. R., D. L. Horner, and J. S. Logan. (1989) The growth hormone-binding protein in rat serum is an alternatively spliced form of the rat growth hormone receptor. *Genes & Development* **3**:1199-205.
18. Baxter, R. C. (1985) Measurement of growth hormone and prolactin receptor turnover in rat liver. *Endocrinology* **117**:650-5.
19. Behncken, S. N., N. Billestrup, R. Brown, J. Amstrup, B. Conway-Campbell, and M. J. Waters. (2000) Growth hormone (GH)-independent dimerization of GH receptor by a leucine zipper results in constitutive activation. *J Biol Chem* **275**:17000-7.
20. Billestrup, N., P. Bouchelouche, G. Allevato, M. Ilondo, and J. H. Nielsen. (1995) Growth hormone receptor C-terminal domains required for growth hormone-induced intracellular free Ca<sup>2+</sup> oscillations and gene transcription. *Proc Natl Acad Sci U S A* **92**:2725-9.

21. Billestrup, N., and J. H. Nielsen. (1991) The stimulatory effect of growth hormone, prolactin, and placental lactogen on beta-cell proliferation is not mediated by insulin-like growth factor-I. *Endocrinology* **129**:883-8.
22. Bittner, M., P. Meltzer, Y. Chen, Y. Jiang, E. Seftor, M. Hendrix, M. Radmacher, R. Simon, Z. Yakhini, A. Ben-Dor, N. Sampas, E. Dougherty, E. Wang, F. Marincola, C. Gooden, J. Lueders, A. Glatfelter, P. Pollock, J. Carpten, E. Gillanders, D. Leja, K. Dietrich, C. Beaudry, M. Berens, D. Alberts, and V. Sondak. (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* **406**:536-40.
23. Burnside, J., S. S. Liou, and L. A. Cogburn. (1991) Molecular cloning of the chicken growth hormone receptor complementary deoxyribonucleic acid: mutation of the gene in sex-linked dwarf chickens. *Endocrinology* **128**:3183-92.
24. Callard, D., B. Lescure, and L. Mazzolini. (1994) A method for the elimination of false positives generated by the mRNA differential display technique. *Biotechniques* **16**:1096-7, 1100-3.
25. Campbell, G. S., D. J. Meyer, R. Raz, D. E. Levy, J. Schwartz, and C. Carter-Su. (1995) Activation of acute phase response factor (APRF)/Stat3 transcription factor by growth hormone. *J Biol Chem* **270**:3974-9.
26. Campbell, G. S., L. Pang, T. Miyasaka, A. R. Saltiel, and C. Carter-Su. (1992) Stimulation by growth hormone of MAP kinase activity in 3T3-F442A fibroblasts. *J Biol Chem* **267**:6074-80.
27. Carter-Su, C., J. Schwartz, and L. S. Smit. (1996) Molecular mechanism of growth hormone action. *Annu Rev Physiol* **58**:187-207.
28. Carter-Su, C., J. R. Stubbart, X. Y. Wang, S. E. Stred, L. S. Argetsinger, and J. A. Shafer. (1989) Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. *J Biol Chem* **264**:18654-61.
29. Chen, W. Y., N. Y. Chen, J. Yun, T. E. Wagner, and J. J. Kopchick. (1994) In vitro and in vivo studies of antagonistic effects of human growth hormone analogs. *J Biol Chem* **269**:15892-7.
30. Chomczynski, P., and N. Sacchi. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem* **162**:156-9.
31. Chow, J. C., P. R. Ling, Z. Qu, L. Laviola, A. Ciccarone, B. R. Bistran, and R. J. Smith. (1996) Growth hormone stimulates tyrosine phosphorylation of JAK2 and STAT5, but not insulin receptor substrate-1 or SHC proteins in liver and skeletal muscle of normal rats in vivo. *Endocrinology* **137**:2880-6.

32. Chowdhary, B. P., H. Ellegren, M. Johansson, L. Andersson, and I. Gustavsson. (1994) In situ hybridization mapping of the growth hormone receptor (GHR) gene assigns a linkage group (C9, FS, GHR, and S0105) to chromosome 16 in pigs. *Mamm Genome* **5**:160-2.
33. Cioffi, J. A., X. Wang, and J. J. Kopchick. (1990) Porcine growth hormone receptor cDNA sequence. *Nucleic Acids Res* **18**:6451.
34. Clackson, T., M. H. Ultsch, J. A. Wells, and A. M. de Vos. (1998) Structural and functional analysis of the 1:1 growth hormone:receptor complex reveals the molecular basis for receptor affinity. *J Mol Biol* **277**:1111-28.
35. Clark, E. A., T. R. Golub, E. S. Lander, and R. O. Hynes. (2000) Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406**:532-5.
36. Cohnen, S. J., D. Sanden, N. A. Cacalano, A. Yoshimura, A. Mui, T. S. Migone, and J. A. Johnston. (1999) SOCS-3 is tyrosine phosphorylated in response to interleukin-2 and suppresses STAT5 phosphorylation and lymphocyte proliferation. *Mol Cell Biol* **19**:4980-8.
37. Coux, O., K. Tanaka, and A. L. Goldberg. (1996) Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* **65**:801-47.
38. Cunningham, B. C., M. Ultsch, A. M. De Vos, M. G. Mulkerrin, K. R. Clauser, and J. A. Wells. (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* **254**:821-5.
39. Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**:1415-21.
40. Dastot, F., M. L. Sobrier, P. Duquesnoy, B. Duriez, M. Goossens, and S. Amselem. (1996) Alternatively spliced forms in the cytoplasmic domain of the human growth hormone (GH) receptor regulate its ability to generate a soluble GH-binding protein. *Proc Natl Acad Sci U S A* **93**:10723-8.
41. Daughaday, W. H., K. Hall, M. S. Raben, W. D. Salmon, Jr., J. L. v. d. Brande, and J. J. v. Wyk. (1972) Somatomedin: proposed designation for sulphation factor. *Nature* **235**:107.
42. De Sepulveda, P., S. Ilangumaran, and R. Rottapel. (2000) Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation. *J Biol Chem* **275**:14005-8.

43. de Vos, A. M., M. Ultsch, and A. A. Kossiakoff. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science* **255**:306-12.
44. Dey, B. R., S. L. Spence, P. Nissley, and R. W. Furlanetto. (1998) Interaction of human suppressor of cytokine signaling (SOCS)-2 with the insulin-like growth factor-I receptor. *J Biol Chem* **273**:24095-101.
45. Doglio, A., C. Dani, P. Grimaldi, and G. Ailhaud. (1989) Growth hormone stimulates c-fos gene expression by means of protein kinase C without increasing inositol lipid turnover. *Proc Natl Acad Sci U S A* **86**:1148-52.
46. Donahue, L. R., and W. G. Beamer. (1993) Growth hormone deficiency in 'little' mice results in aberrant body composition, reduced insulin-like growth factor-I and insulin-like growth factor-binding protein-3 (IGFBP-3), but does not affect IGFBP-2, -1 or -4. *J Endocrinol* **136**:91-104.
47. Durbin, J. E., R. Hackenmiller, M. C. Simon, and D. E. Levy. (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* **84**:443-50.
48. Eden, S., J. Schwartz, and J. L. Kostyo. (1982) Effects of preincubation on the ability of rat adipocytes to bind and respond to growth hormone. *Endocrinology* **111**:1505-12.
49. Eicher, E. M., and B. K. Lee. (1991) Growth hormone receptor (Ghr) and hemoglobin alpha-chain pseudogene 3 (Hba-ps3) map proximal to the myelocytomatosis oncogene (Myc) on mouse chromosome 15. *Mamm Genome* **1**:57-8.
50. Emanuelli, B., P. Peraldi, C. Filloux, D. Sawka-Verhelle, D. Hilton, and E. Van Obberghen. (2000) SOCS-3 is an insulin-induced negative regulator of insulin signaling. *J Biol Chem* **275**:15985-91.
51. Emili, A. Q., and G. Cagney. (2000) Large-scale functional analysis using peptide or protein arrays. *Nat Biotechnol* **18**:393-7.
52. Endo, T. A., M. Masuhara, M. Yokouchi, R. Suzuki, H. Sakamoto, K. Mitsui, A. Matsumoto, S. Tanimura, M. Ohtsubo, H. Misawa, T. Miyazaki, N. Leonor, T. Taniguchi, T. Fujita, Y. Kanakura, S. Komiya, and A. Yoshimura. (1997) A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387**:921-4.
53. Eriksson, H., M. Ridderstrale, and H. Tornqvist. (1995) Tyrosine phosphorylation of the growth hormone (GH) receptor and Janus tyrosine kinase-2 is involved in the insulin-like actions of GH in primary rat adipocytes. *Endocrinology* **136**:5093-101.

54. Fain, J. N., J. H. Ihle, and S. W. Bahouth. (1999) Stimulation of lipolysis but not of leptin release by growth hormone is abolished in adipose tissue from Stat5a and b knockout mice. *Biochem Biophys Res Commun* **263**:201-5.
55. Fernandez, L., A. Flores-Morales, O. Lahuna, D. Sliva, G. Norstedt, L. A. Haldosen, A. Mode, and J. A. Gustafsson. (1998) Desensitization of the growth hormone-induced Janus kinase 2 (Jak 2)/signal transducer and activator of transcription 5 (Stat5)-signaling pathway requires protein synthesis and phospholipase C. *Endocrinology* **139**:1815-24.
56. Frank, S. J., G. Gilliland, A. S. Kraft, and C. S. Arnold. (1994) Interaction of the growth hormone receptor cytoplasmic domain with the JAK2 tyrosine kinase. *Endocrinology* **135**:2228-39.
57. Frick, G. P., and H. M. Goodman. (1992) Characterization of the short isoform of the growth hormone receptor synthesized by rat adipocytes. *Endocrinology* **131**:3083-90.
58. Frick, G. P., L. R. Tai, W. R. Baumbach, and H. M. Goodman. (1998) Tissue distribution, turnover, and glycosylation of the long and short growth hormone receptor isoforms in rat tissues. *Endocrinology* **139**:2824-30.
59. Frick, G. P., L. R. Tai, H. M. Goodman, and o. P. U. o. M. M. S. W. Department. (1994) Subcellular distribution of the long and short isoforms of the growth hormone (GH) receptor in rat adipocytes: both isoforms participate in specific binding of GH. *Endocrinology* **134**:307-14.
60. Fuh, G., B. C. Cunningham, R. Fukunaga, S. Nagata, D. V. Goeddel, and J. A. Wells. (1992) Rational design of potent antagonists to the human growth hormone receptor. *Science* **256**:1677-80.
61. Ganguly, T. C., M. L. O'Brien, S. J. Karpen, J. F. Hyde, F. J. Suchy, and M. Vore. (1997) Regulation of the rat liver sodium-dependent bile acid cotransporter gene by prolactin. Mediation of transcriptional activation by Stat5. *J Clin Invest* **99**:2906-14.
62. Gaur, S., M. E. Morton, G. P. Frick, and H. M. Goodman. (1998) Growth hormone regulates the distribution of L-type calcium channels in rat adipocyte membranes. *Am J Physiol* **275**:C505-14.
63. Gaur, S., H. Yamaguchi, and H. M. Goodman. (1996) Growth hormone regulates cytosolic free calcium in rat fat cells by maintaining L-type calcium channels. *Am J Physiol* **270**:C1478-84.
64. Gebert, C. A., S. H. Park, and D. J. Waxman. (1997) Regulation of signal transducer and activator of transcription (STAT) 5b activation by the temporal pattern of growth hormone stimulation. *Mol Endocrinol* **11**:400-14.

65. Gebert, C. A., S. H. Park, and D. J. Waxman. (1999) Termination of growth hormone pulse-induced STAT5b signaling. *Mol Endocrinol* **13**:38-56.
66. Godowski, P. J., D. W. Leung, L. R. Meacham, J. P. Galgani, R. Hellmiss, R. Keret, P. S. Rotwein, J. S. Parks, Z. Laron, and W. I. Wood. (1989) Characterization of the human growth hormone receptor gene and demonstration of a partial gene deletion in two patients with Laron- type dwarfism. *Proc Natl Acad Sci U S A* **86**:8083-7.
67. Goodman, H. (1994) Basic Medical Endocrinology, Second Edition, p. 225-238, Raven Press Series in Physiology. Raven Pree, New York.
68. Goodman, H. (1965) Effects of growth hormone on glucose metabolism in adipose tissue. *Endocrinology* **76**:1134-1170.
69. Goodman, H. M. (1968) Growth hormone and the metabolism of carbohydrate and lipid in adipose tissue. *Ann N Y Acad Sci* **148**:419-40.
70. Goodman, H. M. (1981) Separation of early and late responses of adipose tissue to growth hormone. *Endocrinology* **109**:120-9.
71. Goodman, H. M., and V. Coiro. (1981) Induction of sensitivity to the insulin-like action of growth hormone in normal rat adipose tissue. *Endocrinology* **108**:113-9.
72. Goodman, H. M., and J. Schwartz. (1994) Growth hormone and lipid metabolism, p. 211-231, Handbook of Physiology, vol. IV. American Physiology Society, Washington.
73. Goodman, H. M., L. R. Tai, and S. R. Chipkin. (1990) The isoquinoline sulfonamide inhibitors of protein phosphorylation, H- 7, H-8, and HA-1004, also inhibit RNA synthesis: studies on responses of adipose tissue to growth hormone. *Endocrinology* **126**:441-50.
74. Gorin, E., and H. M. Goodman. (1985) Turnover of growth hormone receptors in rat adipocytes. *Endocrinology* **116**:1796-805.
75. Gorin, E., G. Grichting, and H. M. Goodman. (1984) Binding and degradation of [125I]human growth hormone in rat adipocytes. *Endocrinology* **115**:467-75.
76. Gouilleux, F., H. Wakao, M. Mundt, and B. Groner. (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J* **13**:4361-9.
77. Gregorieff, A., S. Pyronnet, N. Sonenberg, and A. Veillette. (2000) Regulation of SOCS-1 expression by translational repression. *J Biol Chem* **275**:21596-604.

78. Grichting, G., L. K. Levy, and H. M. Goodman. (1983) Relationship between binding and biological effects of human growth hormone in rat adipocytes. *Endocrinology* **113**:1111-20.
79. Gronowski, A. M., Z. Zhong, Z. Wen, M. J. Thomas, J. E. Darnell, Jr., and P. Rotwein. (1995) In vivo growth hormone treatment rapidly stimulates the tyrosine phosphorylation and activation of Stat3. *Mol Endocrinol* **9**:171-7.
80. Gurland, G., G. Ashcom, B. H. Cochran, and J. Schwartz. (1990) Rapid events in growth hormone action. Induction of c-fos and c-jun transcription in 3T3-F442A preadipocytes. *Endocrinology* **127**:3187-95.
81. Hackett, R. H., Y. D. Wang, S. Sweitzer, G. Feldman, W. I. Wood, and A. C. Lerner. (1997) Mapping of a cytoplasmic domain of the human growth hormone receptor that regulates rates of inactivation of Jak2 and Stat proteins. *J Biol Chem* **272**:11128-32.
82. Hansen, J. A., L. H. Hansen, X. Wang, J. J. Kopchick, F. Gouilleux, B. Groner, J. H. Nielsen, A. Moldrup, E. D. Galsgaard, and N. Billestrup. (1997) The role of GH receptor tyrosine phosphorylation in Stat5 activation. *J Mol Endocrinol* **18**:213-21.
83. Hansen, J. A., K. Lindberg, D. J. Hilton, J. H. Nielsen, and N. Billestrup. (1999) Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. *Mol Endocrinol* **13**:1832-43.
84. Hansen, L. H., X. Wang, J. J. Kopchick, P. Bouchelouche, J. H. Nielsen, E. D. Galsgaard, and N. Billestrup. (1996) Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and Stat5 activation. *J Biol Chem* **271**:12669-73.
85. Harding, P. A., X. Wang, S. Okada, W. Y. Chen, W. Wan, and J. J. Kopchick. (1996) Growth hormone (GH) and a GH antagonist promote GH receptor dimerization and internalization. *J Biol Chem* **271**:6708-12.
86. Hauser, S. D., M. F. McGrath, R. J. Collier, and G. G. Krivi. (1990) Cloning and in vivo expression of bovine growth hormone receptor mRNA. *Mol Cell Endocrinol* **72**:187-200.
87. Hellgren, G., J. O. Jansson, L. M. Carlsson, and B. Carlsson. (1999) The growth hormone receptor associates with Jak1, Jak2 and Tyk2 in human liver. *Growth Horm IGF Res* **9**:212-8.
88. Herrington, J., L. S. Smit, J. Schwartz, and C. Carter-Su. (2000) The role of STAT proteins in growth hormone signaling. *Oncogene* **19**:2585-97.



89. Hicke, L. (1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *FASEB J* 11:1215-26.
90. Hoeffler, J. P., S. A. Hicks, and L. S. Frawley. (1987) Existence of somatotrope subpopulations which are differentially responsive to insulin-like growth factor I and somatostatin. *Endocrinology* 120:1936-41.
91. Ihle, J. N., W. Thierfelder, S. Teglund, D. Stravapodis, D. Wang, J. Feng, and E. Parganas. (1998) Signaling by the cytokine receptor superfamily. *Ann N Y Acad Sci* 865:1-9.
92. Ihle, J. N., B. A. Witthuhn, F. W. Quelle, K. Yamamoto, and O. Silvennoinen. (1995) Signaling through the hematopoietic cytokine receptors. *Annu Rev Immunol* 13:369-98.
93. Ilondo, M. M., A. B. Damholt, B. A. Cunningham, J. A. Wells, P. De Meyts, and R. M. Shymko. (1994) Receptor dimerization determines the effects of growth hormone in primary rat adipocytes and cultured human IM-9 lymphocytes. *Endocrinology* 134:2397-403.
94. Ilondo, M. M., P. De Meyts, and P. Bouchelouche. (1994) Human growth hormone increases cytosolic free calcium in cultured human IM-9 lymphocytes: a novel mechanism of growth hormone transmembrane signalling. *Biochem Biophys Res Commun* 202:391-7.
95. Imaki, T., T. Shibasaki, A. Masuda, M. Hotta, N. Yamauchi, H. Demura, K. Shizume, I. Wakabayashi, and N. Ling. (1986) The effect of glucose and free fatty acids on growth hormone (GH)-releasing factor-mediated GH secretion in rats. *Endocrinology* 118:2390-4.
96. Isaksson, O. G., J. O. Jansson, and I. A. Gause. (1982) Growth hormone stimulates longitudinal bone growth directly. *Science* 216:1237-9.
97. Jiao, H., K. Berrada, W. Yang, M. Tabrizi, L. C. Platanius, and T. Yi. (1996) Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol* 16:6985-92.
98. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. (1996) Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4:313-9.
99. Kaplan, M. H., Y. L. Sun, T. Hoey, and M. J. Grusby. (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-7.

100. Karlsson, H., J. A. Gustafsson, and A. Mode. (1999) Cis desensitizes GH induced Stat5 signaling in rat liver cells. *Mol Cell Endocrinol* **154**:37-43.
101. Kim, S. O., J. Jiang, W. Yi, G. S. Feng, S. J. Frank, and o. M. U. o. A. a. B. U. S. A. Department. (1998) Involvement of the Src homology 2-containing tyrosine phosphatase SHP-2 in growth hormone signaling. *J Biol Chem* **273**:2344-54.
102. Kingston, R. E. (ed.). (1988) Transfection of DNA into eukaryotic cells, vol. 2. John Wiley & Sons, New York, Chichestr, Brisbane, Toronto, Singapore.
103. Klingmuller, U., U. Lorenz, L. C. Cantley, B. G. Neel, and H. F. Lodish. (1995) Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* **80**:729-38.
104. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-5.
105. Laron, Z., A. Pertzalan, and S. Mannheimer. (1966) Genetic pituitary dwarfism with high serum concentration of growth hormone-a new born error of metabolism? *Isr. J. Med. Sci.* **2**:152-155.
106. Legraverend, C., A. Mode, S. Westin, A. Strom, H. Eguchi, P. G. Zaphiropoulos, and J. A. Gustafsson. (1992) Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion. *Mol Endocrinol* **6**:259-66.
107. Leung, D. W., S. A. Spencer, G. Cachianes, R. G. Hammonds, C. Collins, W. J. Henzel, R. Barnard, M. J. Waters, and W. I. Wood. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. *Nature* **330**:537-43.
108. Liang, P., and A. B. Pardee. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**:967-71.
109. Liu, J. L., S. Yakar, and D. LeRoith. (2000) Conditional knockout of mouse insulin-like growth factor-1 gene using the Cre/loxP system. *Proc Soc Exp Biol Med* **223**:344-51.
110. Liu, X., G. W. Robinson, F. Gouilleux, B. Groner, and L. Hennighausen. (1995) Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc Natl Acad Sci U S A* **92**:8831-5.
111. Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, A. Wynshaw-Boris, and L. Hennighausen. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* **11**:179-86.

112. Lobie, P. E., G. Allevato, J. H. Nielsen, G. Norstedt, and N. Billestrup. (1995) Requirement of tyrosine residues 333 and 338 of the growth hormone (GH) receptor for selected GH-stimulated function. *J Biol Chem* **270**:21745-50.
113. Luo, G., and L. Yu-Lee. (2000) Stat5b inhibits NFkappaB-mediated signaling. *Mol Endocrinol* **14**:114-23.
114. Marine, J. C., C. McKay, D. Wang, D. J. Topham, E. Parganas, H. Nakajima, H. Pendeville, H. Yasukawa, A. Sasaki, A. Yoshimura, and J. N. Ihle. (1999) SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell* **98**:617-27.
115. Marine, J. C., D. J. Topham, C. McKay, D. Wang, E. Parganas, D. Stravopodis, A. Yoshimura, and J. N. Ihle. (1999) SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* **98**:609-16.
116. Marrero, I., A. K. Green, P. H. Cobbold, and C. J. Dixon. (1996) Bovine growth hormone induces oscillations in cytosolic free Ca<sup>2+</sup> in single rat hepatocytes. *Biochem J* **313**:525-8.
117. Masuhara, M., H. Sakamoto, A. Matsumoto, R. Suzuki, H. Yasukawa, K. Mitsui, T. Wakioka, S. Tanimura, A. Sasaki, H. Misawa, M. Yokouchi, M. Ohtsubo, and A. Yoshimura. (1997) Cloning and characterization of novel CIS family genes. *Biochem Biophys Res Commun* **239**:439-46.
118. Mathews, L. S., B. Enberg, and G. Norstedt. (1989) Regulation of rat growth hormone receptor gene expression. *J Biol Chem* **264**:9905-10.
119. Mathews, L. S., G. Norstedt, and R. D. Palmiter. (1986) Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc Natl Acad Sci U S A* **83**:9343-7.
120. Matsumoto, A., M. Masuhara, K. Mitsui, M. Yokouchi, M. Ohtsubo, H. Misawa, A. Miyajima, and A. Yoshimura. (1997) CIS, a cytokine inducible SH2 protein, is a target of the JAK-STAT5 pathway and modulates STAT5 activation. *Blood* **89**:3148-54.
121. Matsumoto, A., Y. Seki, M. Kubo, S. Ohtsuka, A. Suzuki, I. Hayashi, K. Tsuji, T. Nakahata, M. Okabe, S. Yamada, and A. Yoshimura. (1999) Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. *Mol Cell Biol* **19**:6396-407.
122. Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber. (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* **84**:431-42.

123. Meyer, D. J., G. S. Campbell, B. H. Cochran, L. S. Argetsinger, A. C. Lerner, D. S. Finbloom, C. Carter-Su, and J. Schwartz. (1994) Growth hormone induces a DNA binding factor related to the interferon-stimulated 91-kDa transcription factor. *J Biol Chem* **269**:4701-4.
124. Miller, J. D., G. S. Tannenbaum, E. Colle, and H. J. Guyda. (1982) Daytime pulsatile growth hormone secretion during childhood and adolescence. *J Clin Endocrinol Metab* **55**:989-94.
125. Moller, C., A. Hansson, B. Enberg, P. E. Lobie, and G. Norstedt. (1992) Growth hormone (GH) induction of tyrosine phosphorylation and activation of mitogen-activated protein kinases in cells transfected with rat GH receptor cDNA. *J Biol Chem* **267**:23403-8.
126. Mou, L., H. Miller, J. Li, E. Wang, and L. Chalifour. (1994) Improvements to the differential display method for gene analysis. *Biochem Biophys Res Commun* **199**:564-9.
127. Murakami, M., M. Narazaki, M. Hibi, H. Yawata, K. Yasukawa, M. Hamaguchi, T. Taga, and T. Kishimoto. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci U S A* **88**:11349-53.
128. Murphy, L. J., and L. Lazarus. (1984) The mouse fibroblast growth hormone receptor: ligand processing and receptor modulation and turnover. *Endocrinology* **115**:1625-32.
129. Naka, T., M. Narazaki, M. Hirata, T. Matsumoto, S. Minamoto, A. Aono, N. Nishimoto, T. Kajita, T. Taga, K. Yoshizaki, S. Akira, and T. Kishimoto. (1997) Structure and function of a new STAT-induced STAT inhibitor. *Nature* **387**:924-9.
130. Narazaki, M., M. Fujimoto, T. Matsumoto, Y. Morita, H. Saito, T. Kajita, K. Yoshizaki, T. Naka, and T. Kishimoto. (1998) Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling. *Proc Natl Acad Sci U S A* **95**:13130-4.
131. Nicholson, S. E., D. De Souza, L. J. Fabri, J. Corbin, T. A. Willson, J. G. Zhang, A. Silva, M. Asimakis, A. Farley, A. D. Nash, D. Metcalf, D. J. Hilton, N. A. Nicola, and M. Baca. (2000) Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc Natl Acad Sci U S A* **97**:6493-8.
132. Nicholson, S. E., and D. J. Hilton. (1998) The SOCS proteins: a new family of negative regulators of signal transduction. *J Leukoc Biol* **63**:665-8.

133. Nicholson, S. E., T. A. Willson, A. Farley, R. Starr, J. G. Zhang, M. Baca, W. S. Alexander, D. Metcalf, D. J. Hilton, and N. A. Nicola. (1999) Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *EMBO J* 18:375-85.
134. Nilsson, A., J. Isgaard, A. Lindahl, L. Peterson, and O. Isaksson. (1987) Effects of unilateral arterial infusion of GH and IGF-I on tibial longitudinal bone growth in hypophysectomized rats. *Calcif Tissue Int* 40:91-6.
135. Odai, H., K. Sasaki, A. Iwamatsu, T. Nakamoto, H. Ueno, T. Yamagata, K. Mitani, Y. Yazaki, and H. Hirai. (1997) Purification and molecular cloning of SH2- and SH3-containing inositol polyphosphate-5-phosphatase, which is involved in the signaling pathway of granulocyte-macrophage colony-stimulating factor, erythropoietin, and Bcr-Abl. *Blood* 89:2745-56.
136. Odai, H., Y. Hanazono, K. Sasaki, A. Iwamatsu, Y. Yazaki, and H. Hirai. (1997) The signal transduction through Grb2/Ash in hematopoietic cells. *Leukemia* 11 Suppl 3:405-7.
137. Pekhletsy, R. I., B. K. Chernov, and P. M. Rubtsov. (1992) Variants of the 5'-untranslated sequence of human growth hormone receptor mRNA. *Mol Cell Endocrinol* 90:103-9.
138. Pircher, T. J., A. Flores-Morales, A. L. Mui, A. R. Saltiel, G. Norstedt, J. A. Gustafsson, and L. A. Haldosen. (1997) Mitogen-activated protein kinase kinase inhibition decreases growth hormone stimulated transcription mediated by STAT5. *Mol Cell Endocrinol* 133:169-76.
139. Pontiroli, A. E., R. Lanzi, L. D. Monti, E. Sandoli, and G. Pozza. (1991) Growth hormone (GH) autofeedback on GH response to GH-releasing hormone. Role of free fatty acids and somatostatin. *J Clin Endocrinol Metab* 72:492-5.
140. Ram, P. A., S. H. Park, H. K. Choi, and D. J. Waxman. (1996) Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. *J Biol Chem* 271:5929-40.
141. Ram, P. A., and D. J. Waxman. (1997) Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. *J Biol Chem* 272:17694-702.
142. Ram, P. A., and D. J. Waxman. (1999) SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *J Biol Chem* 274:35553-61.

143. Ridderstrale, M., E. Degerman, and H. Tornqvist. (1995) Growth hormone stimulates the tyrosine phosphorylation of the insulin receptor substrate-1 and its association with phosphatidylinositol 3-kinase in primary adipocytes. *J Biol Chem* **270**:3471-4.
144. Ridderstrale, M., and H. Tornqvist. (1994) PI-3-kinase inhibitor Wortmannin blocks the insulin-like effects of growth hormone in isolated rat adipocytes. *Biochem Biophys Res Commun* **203**:306-10.
145. Rodbell, M. (1964) Metabolism of isolated fat cells. I. Effects of hormone on glucose metabolism and lipolysis. *J Biol Chem* **239**:375-380.
146. Rogers, S. A., and M. R. Hammerman. (1989) Growth hormone activates phospholipase C in proximal tubular basolateral membranes from canine kidney. *Proc Natl Acad Sci U S A* **86**:6363-6.
147. Rose-John, S., and P. C. Heinrich. (1994) Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem J* **300**:281-90.
148. Roupas, P., and A. C. Herington. (1988) Intracellular processing of growth hormone receptors by adipocytes in primary culture. *Mol Cell Endocrinol* **57**:93-9.
149. Russell, S. M., and E. M. Spencer. (1985) Local injections of human or rat growth hormone or of purified human somatomedin-C stimulate unilateral tibial epiphyseal growth in hypophysectomized rats. *Endocrinology* **116**:2563-7.
150. Salmon, W. J., and W. Daughaday. (1956) Sulfation factor, a serum component mediating the actions of growth hormone in stimulating incorporation of sulfate into cartilage. *J. Clin. Invest.* **35**:733.
151. Savage, M. O., W. F. Blum, M. B. Ranke, M. C. Postel-Vinay, A. M. Cotterill, K. Hall, P. G. Chatelain, M. A. Preece, and R. G. Rosenfeld. (1993) Clinical features and endocrine status in patients with growth hormone insensitivity (Laron syndrome). *J Clin Endocrinol Metab* **77**:1465-71.
152. Schlechter, N. L., S. M. Russell, S. Greenberg, E. M. Spencer, and C. S. Nicoll. (1986) A direct growth effect of growth hormone in rat hindlimb shown by arterial infusion. *Am J Physiol* **250**:E231-5.
153. Schlechter, N. L., S. M. Russell, E. M. Spencer, and C. S. Nicoll. (1986) Evidence suggesting that the direct growth-promoting effect of growth hormone on cartilage in vivo is mediated by local production of somatomedin. *Proc Natl Acad Sci U S A* **83**:7932-4.

154. Schmitz, J., M. Weissenbach, S. Haan, P. C. Heinrich, and F. Schaper. (2000) SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. *J Biol Chem* **275**:12848-56.
155. Schwartz, Y., and H. M. Goodman. (1990) Refractoriness to the insulin-like effects of growth hormone depends upon calcium. *Endocrinology* **127**:170-6.
156. Schwartz, Y., H. Yamaguchi, and H. M. Goodman. (1992) Growth hormone increases intracellular free calcium in rat adipocytes: correlation with actions on carbohydrate metabolism. *Endocrinology* **131**:772-8.
157. Sekine, N., S. Ullrich, R. Regazzi, W. F. Pralong, and C. B. Wollheim. (1996) Postreceptor signalling of growth hormone and prolactin and their effects in the differentiated insulin-secreting cell line, INS-1. *Endocrinology* **137**:1841-50.
158. Shimoda, K., J. van Deursen, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. Vignali, P. C. Doherty, G. Grosveld, W. E. Paul, and J. N. Ihle. (1996) Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* **380**:630-3.
159. Smal, J., J. Closset, G. Hennen, and P. De Meyts. (1987) Receptor binding properties and insulin-like effects of human growth hormone and its 20 kDa-variant in rat adipocytes. *J Biol Chem* **262**:11071-9.
160. Smal, J., and P. De Meyts. (1987) Role of kinase C in the insulin-like effects of human growth hormone in rat adipocytes. *Biochem Biophys Res Commun* **147**:1232-40.
161. Smit, L. S. (1999) Molecular events in growth hormone-receptor interaction and signaling, p. 445-480. In Kostyo (ed.), *Hand Book of Physiology, Section 7, Volume V: Hormonal Control of Growth*. Oxford University Press, New York Oxford.
162. Smit, L. S., D. J. Meyer, N. Billestrup, G. Norstedt, J. Schwartz, and C. Carter-Su. (1996) The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. *Mol Endocrinol* **10**:519-33.
163. Smit, L. S., J. A. Vanderkuur, A. Stimage, Y. Han, G. Luo, L. Y. Yu-Lee, J. Schwartz, and C. Carter-Su. (1997) Growth hormone-induced tyrosyl phosphorylation and deoxyribonucleic acid binding activity of Stat5A and Stat5B. *Endocrinology* **138**:3426-34.
164. Smith, W. C., J. Kuniyoshi, and F. Talamantes. (1989) Mouse serum growth hormone (GH) binding protein has GH receptor extracellular and substituted transmembrane domains. *Mol Endocrinol* **3**:984-90.

165. Sotiropoulos, A., L. Goujon, G. Simonin, P. A. Kelly, M. C. Postel-Vinay, and J. Finidori. (1993) Evidence for generation of the growth hormone-binding protein through proteolysis of the growth hormone membrane receptor. *Endocrinology* **132**:1863-5.
166. Sotiropoulos, A., S. Moutoussamy, F. Renaudie, M. Clauss, C. Kayser, F. Gouilleux, P. A. Kelly, and J. Finidori. (1996) Differential activation of Stat3 and Stat5 by distinct regions of the growth hormone receptor. *Mol Endocrinol* **10**:998-1009.
167. Sotiropoulos, A., M. Perrot-Applanat, H. Dinerstein, A. Pallier, M. C. Postel-Vinay, J. Finidori, and P. A. Kelly. (1994) Distinct cytoplasmic regions of the growth hormone receptor are required for activation of JAK2, mitogen-activated protein kinase, and transcription. *Endocrinology* **135**:1292-8.
168. Southard, J. N., B. A. Barrett, L. Bikbulatova, Y. Ilkbahar, K. Wu, and F. Talamantes. (1995) Growth hormone (GH) receptor and GH-binding protein messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed in mouse liver and placenta. *Endocrinology* **136**:2913-21.
169. Souza, S. C., G. P. Frick, R. Yip, R. B. Lobo, L. R. Tai and H. M. Goodman (1994) Growth hormone stimulates tyrosine phosphorylation of insulin receptor substrate-1. *J Biol Chem* **269**:30085-8.
170. Starr, R., T. A. Willson, E. M. Viney, L. J. Murray, J. R. Rayner, B. J. Jenkins, T. J. Gonda, W. S. Alexander, D. Metcalf, N. A. Nicola, and D. J. Hilton. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature* **387**:917-21.
171. Stofega, M. R., L. S. Argetsinger, H. Wang, A. Ullrich, and C. Carter-Su. (2000) Negative regulation of growth hormone Receptor/JAK2 signaling by signal regulatory protein alpha. *J Biol Chem* **275**:28222-9.
172. Stofega, M. R., H. Wang, A. Ullrich, and C. Carter-Su. (1998) Growth hormone regulation of SIRP and SHP-2 tyrosyl phosphorylation and association. *J Biol Chem* **273**:7112-7.
173. Strous, G. J., P. van Kerkhof, R. Govers, A. Ciechanover, and A. L. Schwartz. (1996) The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. *EMBO Journal* **15**:3806-12.
174. Strous, G. J., P. van Kerkhof, R. Govers, P. Rotwein, and A. L. Schwartz. (1997) Growth hormone-induced signal transduction depends on an intact ubiquitin system. *J Biol Chem* **272**:40-3.
175. Sundstrom, M., T. Lundqvist, J. Rodin, L. B. Giebel, D. Milligan, and G. Norstedt. (1996) Crystal structure of an antagonist mutant of human growth hormone, G120R, in complex with its receptor at 2.9 Å resolution. *J Biol Chem* **271**:32197-203.



176. Suzuki, R., H. Sakamoto, H. Yasukawa, M. Masuhara, T. Wakioka, A. Sasaki, K. Yuge, S. Komiya, A. Inoue, and A. Yoshimura. (1998) CIS3 and JAB have different regulatory roles in interleukin-6 mediated differentiation and STAT3 activation in M1 leukemia cells. *Oncogene* 17:2271-8.
177. Tannenbaum, G. S., and J. B. Martin. (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* 98:562-70.
178. Tanner, J. W., W. Chen, R. L. Young, G. D. Longmore, and A. S. Shaw. (1995) The conserved box 1 motif of cytokine receptors is required for association with JAK kinases. *J Biol Chem* 270:6523-30.
179. Teglund, S., C. McKay, E. Schuetz, J. M. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J. N. Ihle. (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 93:841-50.
180. Thierfelder, W. E., J. M. van Deursen, K. Yamamoto, R. A. Tripp, S. R. Sarawar, R. T. Carson, M. Y. Sangster, D. A. Vignali, P. C. Doherty, G. C. Grosveld, and J. N. Ihle. (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 382:171-4.
181. Thirone, A. C., C. R. Carvalho, and M. J. Saad. (1999) Growth hormone stimulates the tyrosine kinase activity of JAK2 and induces tyrosine phosphorylation of insulin receptor substrates and Shc in rat tissues. *Endocrinology* 140:55-62.
182. Tollet-Egnell, P., A. Flores-Morales, A. Stavreus-Evers, L. Sahlin, and G. Norstedt. (1999) Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. *Endocrinology* 140:3693-704.
183. Udy, G. B., R. P. Towers, R. G. Snell, R. J. Wilkins, S. H. Park, P. A. Ram, D. J. Waxman, and H. W. Davey. (1997) Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci U S A* 94:7239-44.
184. van Kerkhof, P., R. Govers, C. M. Alves dos Santos, and G. J. Strous. (2000) Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J Biol Chem* 275:1575-80.
185. Vanderkuur, J. A., E. R. Butch, S. B. Waters, J. E. Pessin, K. L. Guan, and C. Carter-Su. (1997) Signaling molecules involved in coupling growth hormone receptor to mitogen-activated protein kinase activation. *Endocrinology* 138:4301-7.
186. VanderKuur, J. A., X. Wang, L. Zhang, G. Allevato, N. Billestrup, and C. Carter-Su. (1995) Growth hormone-dependent phosphorylation of tyrosine 333 and/or 338 of the growth hormone receptor. *J Biol Chem* 270:21738-44.

187. VanderKuur, J. A., X. Wang, L. Zhang, G. S. Campbell, G. Allevato, N. Billestrup, G. Norstedt, and C. Carter-Su. (1994) Domains of the growth hormone receptor required for association and activation of JAK2 tyrosine kinase. *J Biol Chem* **269**:21709-17.
188. Verdier, F., S. Chretien, O. Muller, P. Varlet, A. Yoshimura, S. Gisselbrecht, C. Lacombe, and P. Mayeux. (1998) Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated Cis protein. *J Biol Chem* **273**:28185-90.
189. Vogel, W., R. Lammers, J. Huang, and A. Ullrich. (1993) Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* **259**:1611-4.
190. Wakao, H., F. Gouilleux, and B. Groner. (1994) Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response [published erratum appears in EMBO J 1995 Feb 15;14(4):854-5]. *EMBO J* **13**:2182-91.
191. Wang, X., C. Moller, G. Norstedt, and C. Carter-Su. (1993) Growth hormone-promoted tyrosyl phosphorylation of a 121-kDa growth hormone receptor-associated protein. *J Biol Chem* **268**:3573-9.
192. Wang, X., M. D. Uhler, N. Billestrup, G. Norstedt, F. Talamantes, J. H. Nielsen, and C. Carter-Su. (1992) Evidence for association of the cloned liver growth hormone receptor with a tyrosine kinase. *J Biol Chem* **267**:17390-6.
193. Wang, Y. D., and W. I. Wood. (1995) Amino acids of the human growth hormone receptor that are required for proliferation and Jak-STAT signaling. *Mol Endocrinol* **9**:303-11.
194. Wang<sup>a</sup>, X., C. J. Darus, B. C. Xu, and J. J. Kopchick. (1996) Identification of growth hormone receptor (GHR) tyrosine residues required for GHR phosphorylation and JAK2 and STAT5 activation. *Mol Endocrinol* **10**:1249-60.
195. Wang<sup>a</sup>, X., S. C. Souza, B. Kelder, J. A. Cioffi, and J. J. Kopchick. (1995) A 40-amino acid segment of the growth hormone receptor cytoplasmic domain is essential for GH-induced tyrosine-phosphorylated cytosolic proteins. *J Biol Chem* **270**:6261-6.
196. Wang<sup>a</sup>, X., B. Xu, S. C. Souza, and J. J. Kopchick. (1994) Growth hormone (GH) induces tyrosine-phosphorylated proteins in mouse L cells that express recombinant GH receptors. *Proc Natl Acad Sci U S A* **91**:1391-5.
197. Waxman, D. J. (2000) Growth hormone pulse-activated STAT5 signalling: a unique regulatory mechanism governing sexual dimorphism of liver gene expression. *Novartis Found Symp* **227**:61-74.

198. Wells, J. A. (1996) Binding in the growth hormone receptor complex. *Proc Natl Acad Sci U S A* **93**:1-6.
199. Winston, L. A., and P. J. Bertics. (1992) Growth hormone stimulates the tyrosine phosphorylation of 42- and 45- kDa ERK-related proteins. *J Biol Chem* **267**:4747-51.
200. Xu, B. C., X. Wang, C. J. Darus, and J. J. Kopchick. (1996) Growth hormone promotes the association of transcription factor STAT5 with the growth hormone receptor. *J Biol Chem* **271**:19768-73.
201. Yakar, S., J. L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer, and D. LeRoith. (1999) Normal growth and development in the absence of hepatic insulin-like growth factor I. *Proc Natl Acad Sci U S A* **96**:7324-9.
202. Yi, W., S. O. Kim, J. Jiang, S. H. Park, A. S. Kraft, D. J. Waxman, and S. J. Frank. (1996) Growth hormone receptor cytoplasmic domain differentially promotes tyrosine phosphorylation of signal transducers and activators of transcription 5b and 3 by activated JAK2 kinase. *Mol Endocrinol* **10**:1425-43.
203. Yoon, J. B., S. A. Berry, S. Seelig, and H. C. Towle. (1990) An inducible nuclear factor binds to a growth hormone-regulated gene. *J Biol Chem* **265**:19947-54.
204. Yoshimura, A. (1998) The CIS/JAB family: novel negative regulators of JAK signaling pathways. *Leukemia* **12**:1851-7.
205. Yoshimura, A., T. Ohkubo, T. Kiguchi, N. A. Jenkins, D. J. Gilbert, N. G. Copeland, T. Hara, and A. Miyajima. (1995) A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO Journal* **14**:2816-26.
206. Yu, C. L., Y. J. Jin, and S. J. Burakoff. (2000) Cytosolic tyrosine dephosphorylation of STAT5. Potential role of SHP-2 in STAT5 regulation. *J Biol Chem* **275**:599-604.
207. Zapf, J., and E. Froesch. (1999) Insulin-like growth factor I actions on somatic growth, p. 663-700. In K. J. L. (ed.), *Handbook of Physiology, Section 7 The Endocrine System, Volume V: Hormonal Control of Growth*. Oxford University Press, New York Oxford.
208. Zhang, J. G., A. Farley, S. E. Nicholson, T. A. Willson, L. M. Zugaro, R. J. Simpson, R. L. Moritz, D. Cary, R. Richardson, G. Hausmann, B. J. Kile, S. B. Kent, W. S. Alexander, D. Metcalf, D. J. Hilton, N. A. Nicola, and M. Baca. (1999) The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc Natl Acad Sci U S A* **96**:2071-6.

209. Zhang, Y., J. Jiang, J. J. Kopchick, and S. J. Frank. (1999) Disulfide linkage of growth hormone (GH) receptors (GHR) reflects GH- induced GHR dimerization. Association of JAK2 with the GHR is enhanced by receptor dimerization. *J Biol Chem* **274**:33072-84.
210. Zhou, Y., L. He, and J. J. Kopchick. (1996) Structural comparison of a portion of the rat and mouse growth hormone receptor/binding protein genes. *Gene* **177**:257-9.
211. Zhou, Y., B. C. Xu, H. G. Maheshwari, L. He, M. Reed, M. Lozykowski, S. Okada, L. Cataldo, K. Coschigamo, T. E. Wagner, G. Baumann, and J. J. Kopchick. (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci U S A* **94**:13215-20.
212. Zhu, T., E. L. Goh, D. LeRoith, and P. E. Lobie. (1998) Growth hormone stimulates the formation of a multiprotein signaling complex involving p130(Cas) and CrkII. Resultant activation of c-Jun N- terminal kinase/stress-activated protein kinase (JNK/SAPK). *J Biol Chem* **273**:33864-75.
213. Zhu, T., E. L. Goh, and P. E. Lobie. (1998) Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2. Fak is not required for stat-mediated transcription. *J Biol Chem* **273**:10682-9.
214. Zou, L., L. A. Burmeister, and M. A. Sperling. (1997) Isolation of a liver-specific promoter for human growth hormone receptor gene. *Endocrinology* **138**:1771-4.