5-2-2002

# The modulation and cross-talk between STAT 5 and SOCS-3 in insulin and growth hormone signaling in 3T3-L1 adipocytes.

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# The modulation and cross-talk between STAT 5 and SOCS-3 in insulin and growth hormone signaling in 3T3-L1 adipocytes.

An Undergraduate Thesis

by

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Under the direction of

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Submitted to the Honors College of
Louisiana State University and Agricultural and Mechanical College
In partial fulfillment of the requirements for
Upper Division Honors Distinction in
Biochemistry

May 2, 2002

# INTRODUCTION

According to the National Center for Health Statistics, diabetes is the sixth leading cause of death in the United States, causing nearly 70,000 deaths annually (1). Nearly 90% of diabetics in America suffer from non-insulin dependent diabetes mellitus (NIDDM or type-II diabetes), as opposed to insulin dependent diabetes mellitus (IDDM or type-I diabetes) (2). The World Health Organization projects a 35% increase in the prevalence of type-II diabetes based on current numbers (3). Unlike the type-I disorder, patients suffering from type-II are usually not helped by the administration of insulin. The problem is not an inability to produce insulin, their pancreatic β-cells are often normal, but the three insulin sensitive tissues in the body, fat, skeletal muscle, and liver, become insulin resistant (4). Also, there is an interesting correlation between type-II diabetics and obesity. Nearly 80% of all type-II diabetics are obese (5). Moreover, when these patients lose weight, they also lose the diabetes (6). This connection is why our laboratory chooses to do research on fat cells, or adipocytes.

The binding of insulin to its receptor triggers autophosphorylation on several tyrosine residues present in the cytosolic portion of the molecule. The insulin receptor also tyrosine phosphorylates insulin receptor substrates (IRSs). The IRS proteins dock with the receptor and the phosphorylated residues can act as binding sites for other proteins that contain SH2 domains. Several enzymes and adapter proteins associate with the IRS proteins and are subsequently phosphorylated and activated. These signaling proteins are responsible for the downstream events which follow insulin stimulation. Insulin can activate the phosphatidylinositol 3-kinase (PI-3K) pathway, resulting in protein kinase B and C (PKB and PKC) activation, and the mitogen activated protein kinase (MAPK) pathway (7). A significant portion of the insulin cascade has been elucidated, but there are still many gaps in our understanding of the mechanism.

STATs (Signal Transducers and Activators of Transcription) are a family of transcription factors that our lab has been studying in fat cells. There are seven proteins (STATs 1, 2, 3, 4, 5A, 5B, and 6) in this family of proteins. The binding of several different cytokines to their specific

receptors results in STAT phosphorylation, dimerization, and translocation to the nucleus, where they act to regulate the transcription of particular genes in cell or tissue specific manners (8-13). SOCS (Suppressors of Cytokine Signaling) are a novel family of eight proteins (CIS and SOCS 1-7) that have been shown to be involved in the attenuation of STAT signaling. Our laboratory has characterized these proteins in adipocytes and shown some link between the protein levels and type-II diabetes by studying their regulation in three independent rodent models of type-II diabetes. Further studies have shown that many of the same cytokines that activate STATs, also induce SOCS expression (12, 14-18)(refer to Schematic 1).

STAT 5A and STAT 5B are activated by GH in many cell types. However, some recent studies also suggest that STAT 5B is a physiological substrate of the insulin receptor in 293 EBNA cells over expressing the insulin receptor (19), in mouse liver cells (20), and in Kym-1 rhabdomyosarcoma cells (21). Two studies suggest that insulin activation of STAT 5B is independent of JAK2 kinase, which is normally involved in STAT 5 phosphorylation (21,22). Similar findings have been reported for SOCS-3. A recent study has demonstrated that SOCS-3 mRNA and STAT 5B phosphorylation are induced by insulin in 3T3-L1 adipocytes (23). Additional studies by Van Obberghen and colleagues suggest that SOCS-3 and STAT 5B compete for binding at phosphotyrosine 960 of the insulin receptor. These studies also suggest that insulin phosphorylates SOCS-3 via JAK kinases (24). SOCS-1 and SOCS-6 have also been shown to associate with the insulin receptor and act as potential inhibitors of insulin receptor signaling in rat hepatoma cells (25).

The role of the earliest discovered SOCS has been partly elucidated thanks to gene knockouts in mice. Mice completely deficient of SOCS-1 show a hyper-responsiveness to IFN-γ, suggesting that SOCS-1 is involved in the negative regulation of IFN-γ signaling (26). SOCS-1 has also shown to be involved in T-cell differentiation (27). SOCS-2 knockouts are 30-40% larger in mass than wild-type mice, indicating a prominent role of SOCS-2 in the growth of an organism (27). Studies of SOCS-3 knockout mice established that SOCS-3 was a critical regulator of fetal liver hematopoiesis (28). Further evidence of the importance of this family of proteins is shown by

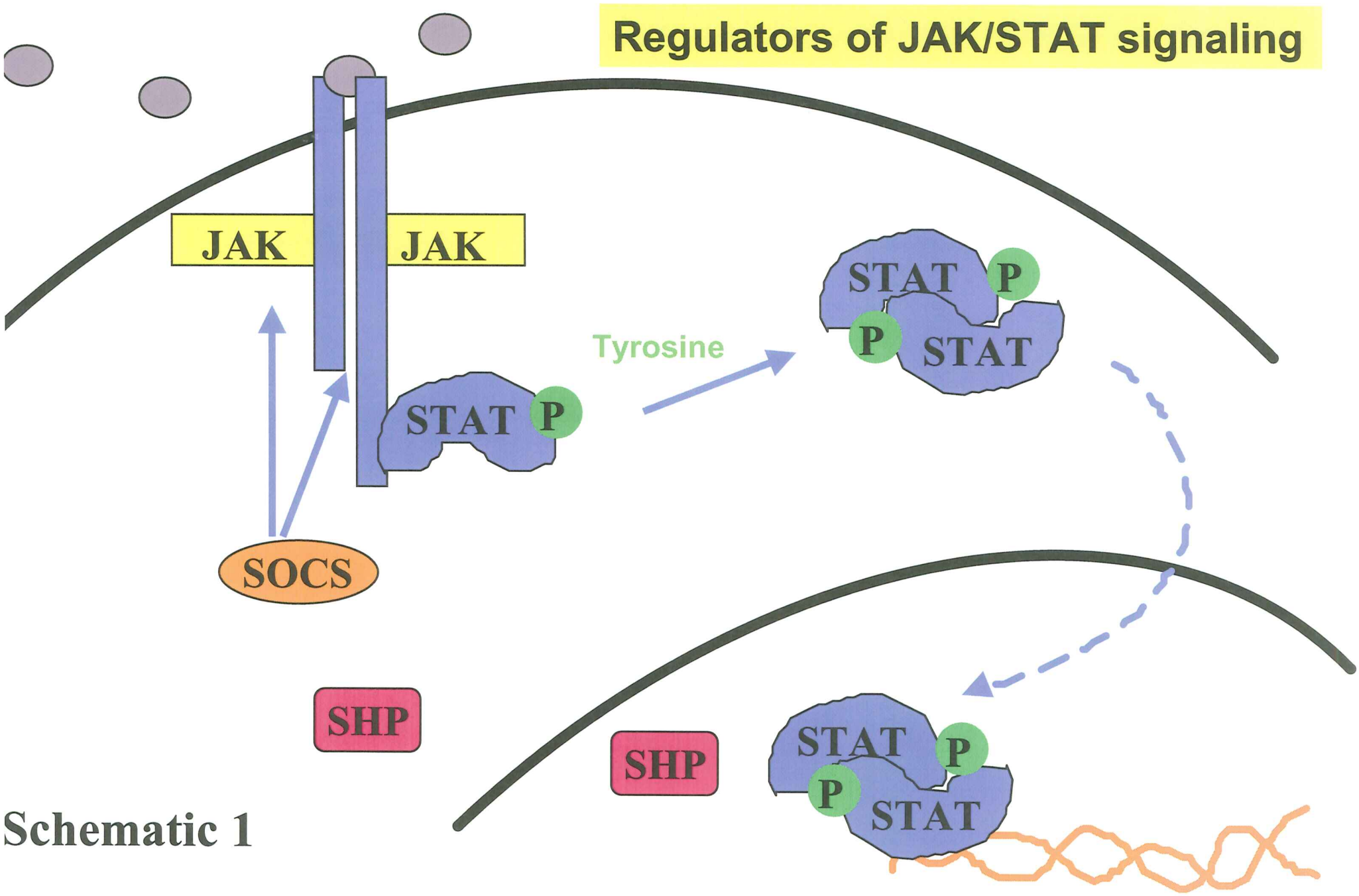
the fact that SOCS-1 and SOCS-3 knockouts are embryonic lethal mutations, and that these studies were done prenatally (26-29).

In order to ascertain the functions of proteins, it is important to investigate their structures. The general construction of the SOCS protein includes a highly variable amino terminus, a central SH2 domain, and a highly conserved, forty amino acid, C-terminal motif now known as the SOCS box (30). More than twenty proteins containing SOCS boxes have been identified (31). The eight proteins which constitute the SOCS family all contain the central SH2 domain. The SH2 domain facilitates direct binding of the SOCS proteins to phosphorylated tyrosines on cytokine receptors or JAKs. This binding incorporates SOCS into the signaling complex where it can block STAT binding sites and/or attenuate the catalytic activity of the JAK (32). The SOCS box has also recently been shown to link the protein with E3 of the ubiquitin pathway and the proteasome (26). Therefore, if the SH2 domain is bound to a phosphorylated JAK or receptor, and the SOCS box is linked to E3 and the proteasome, then it is feasible that the SOCS proteins play a key role in protein turnover, and the degradation of signaling proteins could be the mechanism for SOCS proteins' ability to attenuate cytokine signaling. However, this has yet to be proven in the laboratory.

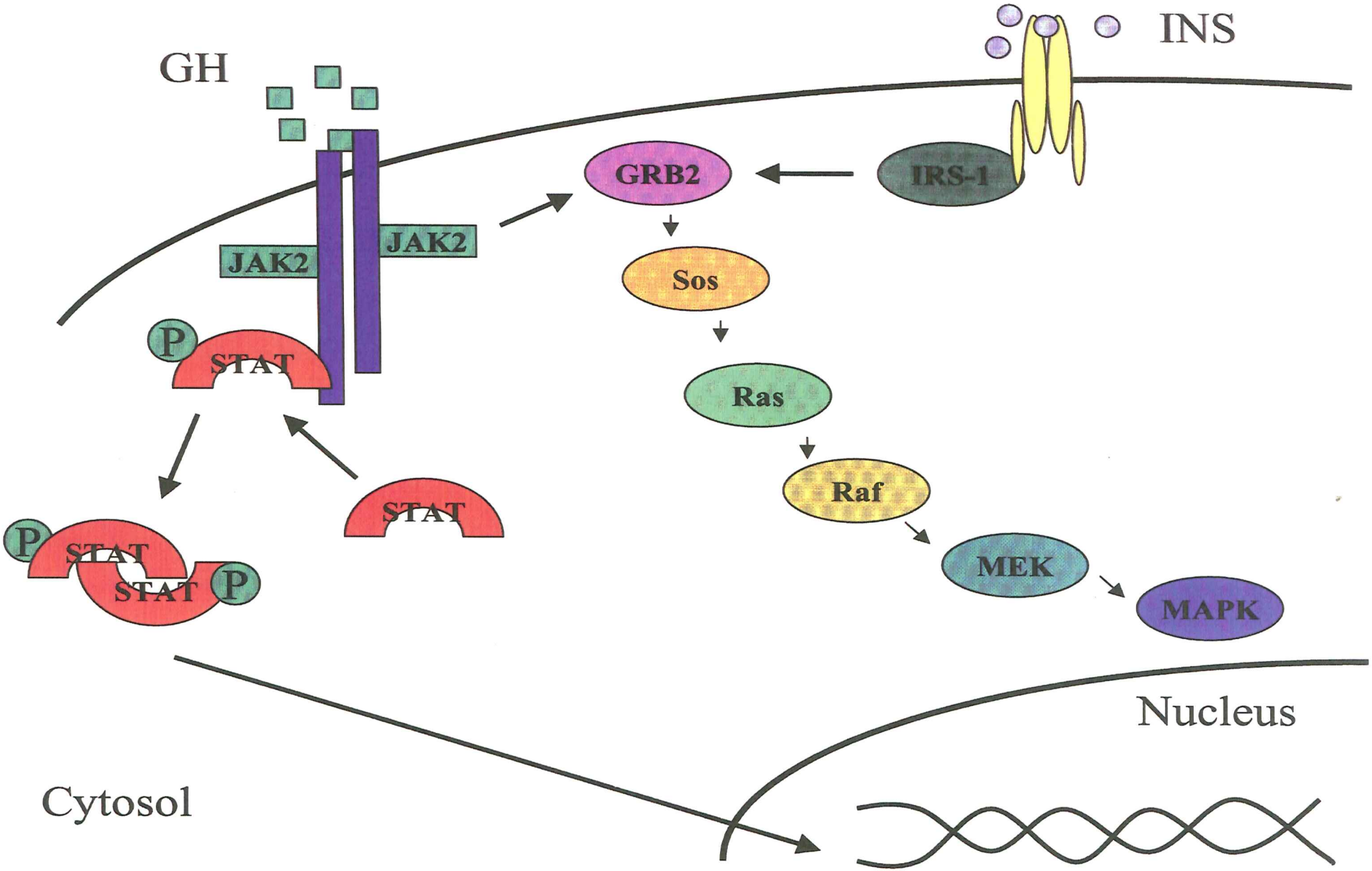
In the past few years, our laboratory has focused on the activation of STAT proteins in cultured and native adipocytes. Our results reveal a very specific activation of STAT 5 proteins in fat cells. More than twenty-five cytokines, growth factors, and hormones were used to treat cultured and native adipocytes, and only GH treatment resulted in STAT 5A and 5B activation (8). GH signaling is initiated by the binding of a single GH molecule to a pair of GH receptors. The dimerization of the receptors leads to the phosphorylation of JAK2, a cytosolic tyrosine kinase that associates with the cytoplasmic domain of the receptor and is thought to modulate all the downstream signaling events from these receptors. The signaling molecules that are recruited and activated by the GHR-JAK2 complex include STATs and the insulin receptor substrates (IRSs) 1 and 2 (26). The recruitment and stimulation of these signaling intermediates leads to the activation of the mitogen activated protein kinase (MAPK) pathway and the

phophatidylinositol-3'-kinase (PI-3K) pathway, and to the release of second messengers such as diacylglycerol, calcium, and nitric oxide (33). Clearly, there appear to be many similarities in the GH and insulin signaling cascades (refer to Schematic 2).

The goal of my project was to compare the effects of GH and insulin on STAT 5 phosphorylation and the induction of SOCS-3 in adipocytes. In addition, we examined the cross talk between these two signaling proteins in 3T3-L1 adipocytes. As expected, GH was a potent inducer of STAT 5A and 5B. However, a variety of insulin treatments had no effect on STAT 5 activation. Interestingly, both GH and insulin were capable of inducing the SOCS-3 transcript in an MAPK independent fashion. GH caused a much stronger induction of SOCS-3 mRNA when compared to insulin, which did not result in the presence of SOCS-3 protein. The weak induction of SOCS-3 by insulin was also insufficient to attenuate GH action. Our studies demonstrate that pre-incubation with GH, but not insulin, inhibits GH signaling. Moreover, inhibition of growth hormone signaling did not require the sustained presence of GH in the culture media or the translation of the SOCS-3 mRNA. The presence of cycloheximide caused a super-induction of SOCS-3 mRNA, but did not affect the ability of GH to activate STAT 5. In summary, our results indicate that the induction of SOCS-3 is not necessary to attenuate GH or insulin signaling in adipocytes, and that STAT 5 is not activated by insulin in 3T3-L1 adipocytes.


Regulators of JAK/STAT signaling
JAK
JAK
STAT
P
Tyrosine
STAT
P
P
STAT
SOCS
SHP
SHP
STAT
P
P
STAT

Schematic 1


GH
INS
GRB2
IRS-1
JAK2
JAK2
Sos
P
STAT
Ras
Raf
STAT
P
STAT
STAT
P
MEK
MAPK
Nucleus
Cytosol

**Schematic 2**

## Experimental Procedures

Materials-Dulbecco's Modified Eagle's Media (DMEM) was purchased from Life Technologies. Bovine and fetal bovine serum (FBS) were obtained from Sigma and Life Technologies, respectively. GH and insulin were purchased from Sigma. The non-phospho STAT antibodies were monoclonal IgGs purchased from BD Transduction Laboratories or polyclonal IgGs from Santa Cruz. A highly phospho-specific polyclonal antibody for STAT 5 (Y694) was purchased from UBI. ERK1/ERK2 was a rabbit polyclonal from Santa Cruz and anti-active MAPK (ERK1/2) was obtained from Promega .

Cell Culture - Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in DMEM with 10% bovine serum. Medium was changed every 48 hours. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, and 1.7 mM insulin. After 48 hours this medium was replaced with DMEM supplemented with 10% FBS, and cells were maintained in this medium until utilized for experimentation.

Preparation of Whole Cell Extracts - Monolayers of 3T3-L1 adipocytes were rinsed with phosphate-buffered saline (PBS) and then harvested in a non-denaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, and 10 mM leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 minutes on ice and centrifuged at 15,000 rpm at 4oC for 15 minutes. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

Preparation of Nuclear/Cytosolic Extracts - Cell monolayers were rinsed with PBS and then harvested in a nuclear homogenization buffer (NHB) containing 20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM MgCl2. Nonidet P-40 was added to a final concentration of 0.15%, and cells were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 1500 rpm for 5 minutes. Supernatants were saved as cytosolic extract, and the nuclear pellets were resuspended in 1/2 volume of NHB and were centrifuged as before. The pellet of intact nuclei was resuspended again in 1/2 of the original volume of NHB and centrifuged again. A small portion of the nuclei was used for Trypan Blue staining to examine the integrity of the nuclei. The majority of the pellet (intact nuclei) was resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 25% glycerol. Nuclei were extracted for 30 minutes on ice and then placed at room temperature for 10 minutes. Two hundred units of DNase1 was added to each sample, tubes were inverted, and incubated an additional 10 minutes at room temperature. Finally, the sample was subjected to centrifugation at 15,000 rpm at 4oC for 30 minutes. Supernatants containing nuclear extracts were analyzed for protein content.

Gel Electrophoresis and Immunoblotting -Proteins were separated in 5%, 7.5%, or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (34) and transferred to nitrocellulose (Biorad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk for 1 hour at room temperature. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

## RESULTS

**Growth hormone and insulin activate MAPK (ERKs 1 and 2), but only growth hormone activates STAT 5 proteins.** Fully differentiated 3T3-L1 adipocytes were treated for 15 minutes with either GH or insulin. The cells were homogenized and fractionated into nuclear and cytosolic extracts. The extracts were then separated by SDS-PAGE and results were visualized by Western blot analysis. As shown in Figure 1, nuclear levels of STAT 5A and 5B were markedly increased in cells treated with growth hormone, while cells treated with insulin showed no difference in STAT 5A and 5B levels when compared to untreated cells. STAT 5 tyrosine phosphorylation was assessed by using a phospho-specific antibody which can recognize both STATs 5A and 5B. Tyrosine phosphorylated STAT 5 proteins were undetectable in both the cytosol and nucleus in untreated and insulin-treated cells, but growth hormone highly induced nuclear levels of phosphorylated STAT 5. The efficacy of the insulin is demonstrated by the activation of MAPK (ERKs 1 and 2). Active MAPK is present in the cytosol and nucleus after acute GH or insulin treatment, whereas the levels of MAPK are unaffected by these treatments. In addition, we have shown that doses of insulin ranging from 1 nM to 10 μM do not effect STAT 5 phosphorylation in 3T3-L1 adipocytes (data not shown).

**The regulation of SOCS-3 mRNA and protein by growth hormone and insulin.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated for various times with either GH or insulin. As shown in Figure 2A, GH treatment results in the induction of SOCS-3 protein. Also, the presence of SOCS-3 protein correlates with the loss of STAT 5 tyrosine phosphorylation. Insulin treatment did not result in the induction of SOCS-3 protein. However, the results in Figure 2B clearly indicate that insulin treatment did result in a notable increase in SOCS-3 mRNA. Insulin-treated cells showed increased levels of SOCS-3 mRNA at 30 minutes and 1 hour which gradually decreased over 8 hours. Compared to insulin, growth hormone was a much more potent inducer of SOCS-3 mRNA. The levels of SOCS-3 mRNA levels were increased at 30 minutes and 1 hour, but to a much higher degree than

resulted from the insulin treatment. Interestingly, the SOCS-3 mRNA disappeared at a quicker rate with GH treatment.

**The role of protein synthesis and active MAPK on the GH and insulin modulation of STAT 5 and SOCS-3.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated for various times with either GH or insulin in the presence of the MEK inhibitor, U0126, or cycloheximide. As shown in Figure 3A, the GH induction of STAT 5 phosphorylation at 30 min or 1 hr was unaffected by the absence of active MAPK or new protein synthesis. The efficacy of the MEK inhibitor is demonstrated by examining the presence of active MAPK in the bottom panel of Figure 3A. Even loading of the samples is demonstrated by examining the expression of STAT 5A. An analysis of SOCS-3 mRNA from this experiment demonstrates that there is very little SOCS-3 mRNA present in the absence of GH or insulin treatment (0 min). At 4 hours, the highest levels of SOCS-3 mRNA are observed in the presence of cycloheximide. As previously shown in Figure 1, a 30 min. or 1 hr. treatment of GH or insulin results in an increase in SOCS-3 mRNA. The induction of SOCS-3 by a 30 min GH or insulin treatment is unaffected by cycloheximide and slightly reduced in the presence of U0126. After a 1 hour treatment, SOCS-3 mRNA is more abundant in cycloheximide treated adipocytes that were exposed to GH or insulin. In addition, there is a slight decline in SOCS-3 mRNA in the presence of the MEK inhibitor.

**Inhibition of MAPK does not inhibit the induction of SOCS-3.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated for various times with either GH in the presence of the MEK inhibitor, U0126, or cycloheximide. As shown in Figure 4B, the presence of U0126 does not substantially effect GH induced STAT 5 phosphorylation. The efficacy of the MEK inhibitor is shown by immunoblotting with the active MAPK antibody and the even loading of protein is demonstrated with the examination of MAPK. An analysis of SOCS-3 mRNA from this experiment clearly demonstrates that cycloheximide treatment results in a significant increase in SOCS-3 mRNA, whereas the MEK inhibitor results in a subtle decrease in SOCS-3 mRNA.

**GH pretreatment inhibits GH induced STAT 5 phosphorylation in a manner that is independent of new protein synthesis or active MAPK.** Whole cell extracts were isolated from fully differentiated 3T3-L1 adipocytes that were treated for various times with GH in the presence or absence of a GH pre-treatment for the times indicated in Figure 5A. As previously shown, acute GH treatment (15 min) resulted in a robust tyrosine phosphorylation of STAT 5 proteins. This stimulation of STAT 5 was inhibited with a GH pretreatment of 2, 8, or 18 hours. The results in Figure 5B demonstrate that the presence of cycloheximide did not affect the ability of a GH pretreatment to inhibit STAT 5 phosphorylation. Moreover, the inhibition of MAPK did not affect the ability of a GH pretreatment to inhibit STAT 5 phosphorylation (Figure 5C).

**The translation of SOCS-3 mRNA is not necessary for the inhibition of GH induced STAT 5 phosphorylation by a GH pretreatment.** The results in Figure 4 demonstrate that SOCS-3 mRNA is super induced by cycloheximide treatment and the results in Figure 5 indicate new protein synthesis is not needed for the attenuation of GH signaling. Therefore, we examined the expression of SOCS-3 mRNA and protein after a GH treatment in the presence of cycloheximide. Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated for various times with either GH in the presence or absence of cycloheximide (CH). As previously shown, acute GH treatment (30 min) results in an increase of STAT 5 activation, MAPK activation and SOCS-3 mRNA. There is no SOCS-3 protein at 4 hour in the presence of CH. Cycloheximide also resulted in an inhibition of SOCS-3 protein at 30 min (data not shown). Interestingly, the SOCS-3 mRNA was induced by CH alone, in the absence of GH stimulation. STAT 5A is shown as even loading control for the protein samples.

**Lack of cross-talk between growth hormone and insulin.** Fully differentiated 3T3-L1 adipocytes were treated for 15 minutes with either GH or insulin after the different pretreatments indicated at the top of each panel. The cells were homogenized and fractionated into nuclear and cytosolic extracts. The extracts were then separated by SDS-PAGE and results were visualized by Western blot analysis. As shown in Figure 7A, acute GH treatment resulted in STAT 5 phosphorylation and nuclear translocation in adipocytes that were not GH pretreated and in cells

that were pretreated with insulin for 3 hrs or 18 hrs. The results in Figure 7B demonstrate that the acute activation of MAPK by insulin is only attenuated by insulin pretreatments, but not by GH pretreatments. The results in Figure 7C demonstrate that GH pretreatment can inhibit acute GH activation of MAPK and STAT 5, but does not effect the acute insulin treatment on MAPK.

## DISCUSSION

The novel findings of my project show the effects that GH and insulin have on the activation of STAT 5 and the induction of SOCS-3 mRNA in 3T3-L1 adipocytes. The present evidence demonstrates that GH, but not insulin, can trigger the phosphorylation and nuclear translocation of STAT 5. Our results also clearly indicate that both insulin and GH can cause an induction of SOCS-3 mRNA, but that GH stimulation results in a stronger induction comparatively. Moreover, only GH treatment results in a detectable increase in SOCS-3 protein. Also presented in this paper is evidence demonstrating that; the attenuation of GH signaling following a pretreatment with GH is independent of protein synthesis and active MAPK (ERKs 1 and 2), that SOCS-3 mRNA induction is independent of ERKs 1 and 2, and that cycloheximide results in the super induction of SOCS-3 mRNA in fat cells.

There are several studies which have shown mRNAs to be superinduced in the presence of cycloheximide (34-36). The results of my study show that cycloheximide causes an induction of SOCS-3 transcript in both the presence and absence of GH. When both cycloheximide and GH are present, the SOCS-3 mRNA signal is stronger and persists in the cell much longer than when treated with GH alone. It is not known whether this increase in induction and persistence is the result of increased transcription or stabilization of SOCS-3 mRNA. However, treatment with cycloheximide results directly in an increased level of SOCS-3 mRNA. Additionally, the enhanced mRNA levels may be indicative of SOCS-3 protein being involved in a negative feedback system regulating its own transcription.

There have been reports published suggesting that some SOCS proteins are able to inhibit insulin signaling. Mooney et al have shown that SOCS-1 and SOCS-6 interact with the insulin receptor and attenuate the activation of Insulin Receptor Substrate-1 (IRS-1) in human hepatoma cells (25). Van Obberghen and colleagues have reported that SOCS-3

can bind to the insulin receptor and decrease insulin induced phosphorylation of IRS-1 in COS-7 cells (37). There is also a study suggesting that SOCS-3 binds to phosphorylated tyrosine 960 of the insulin receptor (24). We have examined an alternate pathway that is stimulated by insulin, resulting in the activation of MAPK (ERKs 1 and 2). Our data indicate that the induction of SOCS-3 mRNA is independent of active MAPK or new protein synthesis. In addition, SOCS-3 protein is not induced by insulin, and therefore is probably not a physiological inhibitor of the insulin signaling pathway. There is still attenuation of insulin signaling in the absence of SOCS-3 in 3T3-L1 adipocytes.

SOCS are critical components of cytokine signaling. The mRNA level of SOCS-1, SOCS-2, SOCS-3, and CIS are all rapidly increased in response to several cytokines. Many of the interleukins, interferon-γ (IFN-γ), leukemia inhibitory factor (LIF), and GH are just some of the cytokines that trigger the induction of SOCS transcripts (27). Their roles in the cytokine signaling pathways include participating in a classical negative feedback circuit. Mechanisms by which the SOCS function in these negative feedback systems include blocking binding sites of STATs, as was shown by Van Obberghen and colleagues (23), inactivating Janus kinases (JAKs), and ubiquitination, and subsequent degradation, of proteins involved in the signaling cascades (27). We have shown that GH treatment results in immediate STAT 5 activation and delayed SOCS-3 protein induction in 3T3-L1 adipocytes. Despite an apparent correlation between STAT 5 deactivation and SOCS-3 induction in response to GH, there is evidence showing that SOCS-3 is not involved in attenuating the GH-induced activation of STAT 5 or MAPK.

It is clear that 3T3-L1 adipocytes lose their ability to respond to both GH and insulin for a period no less than 18 hours following treatment with the same ligand. This could be caused by any number of activities in the cell. The most obvious possibility is the insensitivity of the cell receptor to respond to the ligand in the media. For some reason, the receptor once activated, can not be stimulated again for 18 hours. An inhibitor may be preventing binding of second messengers in the cytoplasm to the receptor, or the receptor

may be endocytosed following activation. This paper has resolved that if there is an inhibitor molecule involved in this attenuation, it is not SOCS-3.

This project was designed to investigate if SOCS-3 could be involved in the attenuation of the GH receptor response following pretreatment with GH and to determine if any cross-talk occurs between GH and insulin signaling. We have clearly shown that the attenuation of GH signaling that occurs after pretreatment is independent of SOCS-3 expression, and also independent of ERK 1 and 2 activation in 3T3-L1 adipocytes. Furthermore, SOCS-3 does not inhibit the insulin induced activation of MAPK in 3T3-L1 adipocytes. Growth hormone treatment activates STAT 5 and facilitates its nuclear translocation, and induces SOCS-3 expression, but has no apparent effect on insulin signaling in 3T3-L1 adipocytes. Insulin stimulation also has no apparent effect on GH signaling either. Therefore, there is no evident cross-talk between the signaling pathways of insulin and growth hormone. To conclude, future investigations regarding SOCS regulation, expression, and stimulation could yield knowledge that leads toward a successful treatment of type II diabetes. These are novel and exciting proteins which have functions we are only beginning to extrapolate.

**Figure 1. Growth hormone, not insulin, induces STATs 5A and 5B tyrosine phosphorylation and nuclear translocation in adipocytes.** Cytosolic and nuclear extracts were isolated from fully differentiated 3T3-L1 adipocytes that were untreated (-) or treated for 15 min. with either 50 nM Insulin (I) or 150 ng/ml of growth hormone (G). One hundred mg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The molecular mass of each protein is indicated to the left of the blot in kilodaltons. The detection system was horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce). This is a representative experiment independently performed three times.

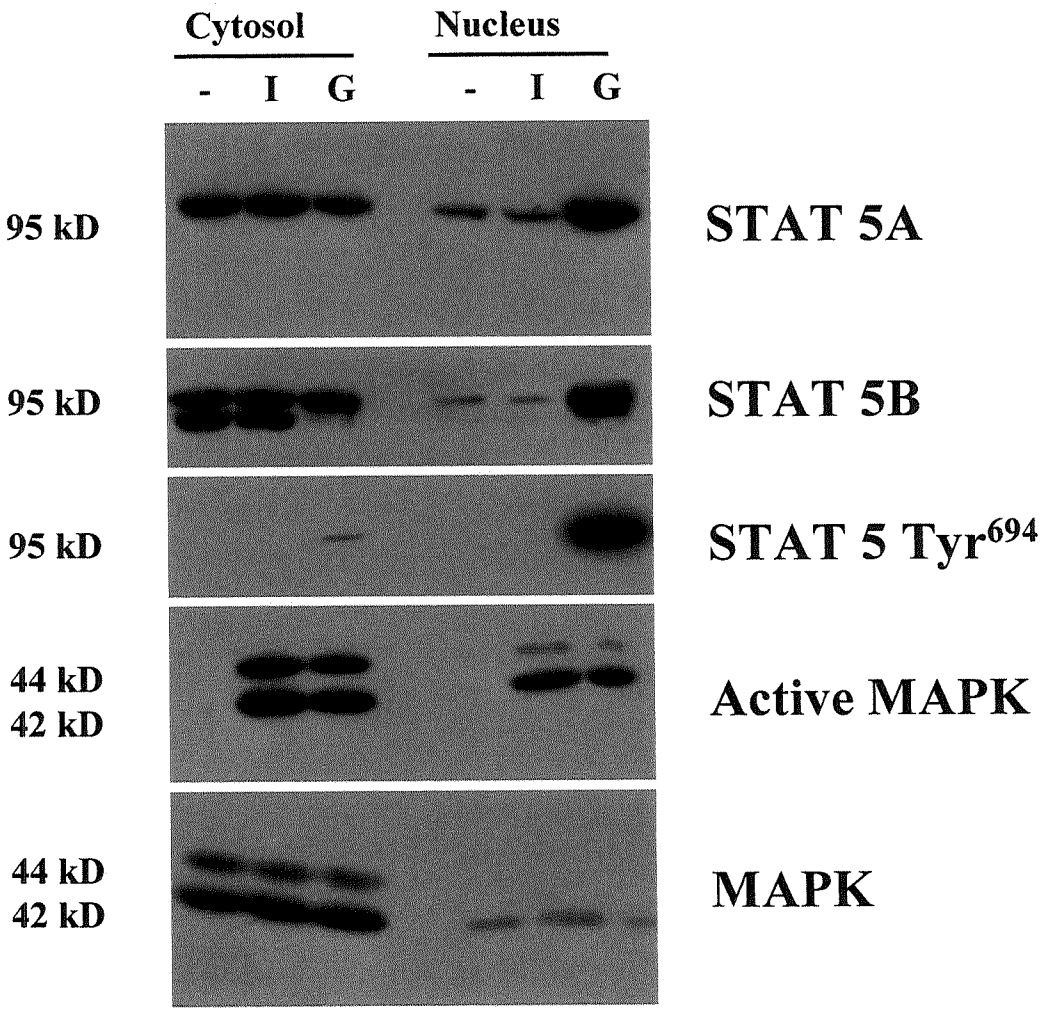
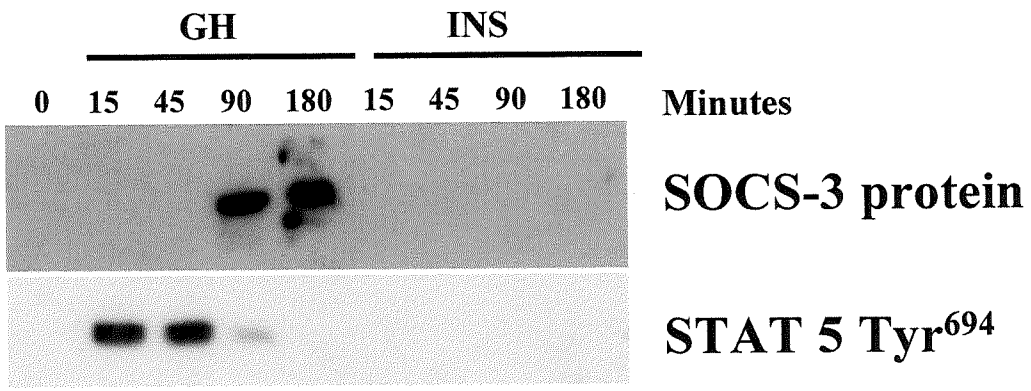

Cytosol
Nucleus
- I G
- I G
95 kD
STAT 5A
95 kD
STAT 5B
95 kD
STAT 5 Tyr694
44 kD
42 kD
Active MAPK
44 kD
42 kD
MAPK

Figure 1

**Figure 2. The induction of SOCS-3 protein and mRNA following treatment with either GH or insulin.** A. Whole cell extracts were isolated from fully differentiated 3T3-L1 adipocytes that were treated with either growth hormone (GH) or insulin (INS) for the times indicated at the top of the figure. One hundred mg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. Samples were processed and results were visualized as described in Figure Legend 1. B. Total RNA was isolated after GH or INS treatment. Fifteen mg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern Blot Analysis. This is a representative experiment independently performed two times.

A.


GH
INS
0 15 45 90 180 15 45 90 180
Minutes
SOCS-3 protein
STAT 5 Tyr694

B.

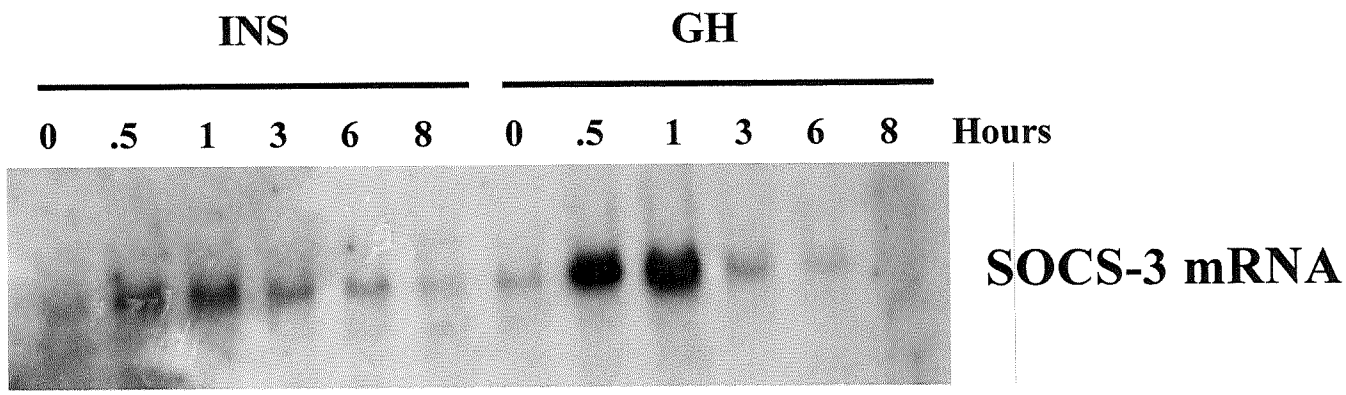
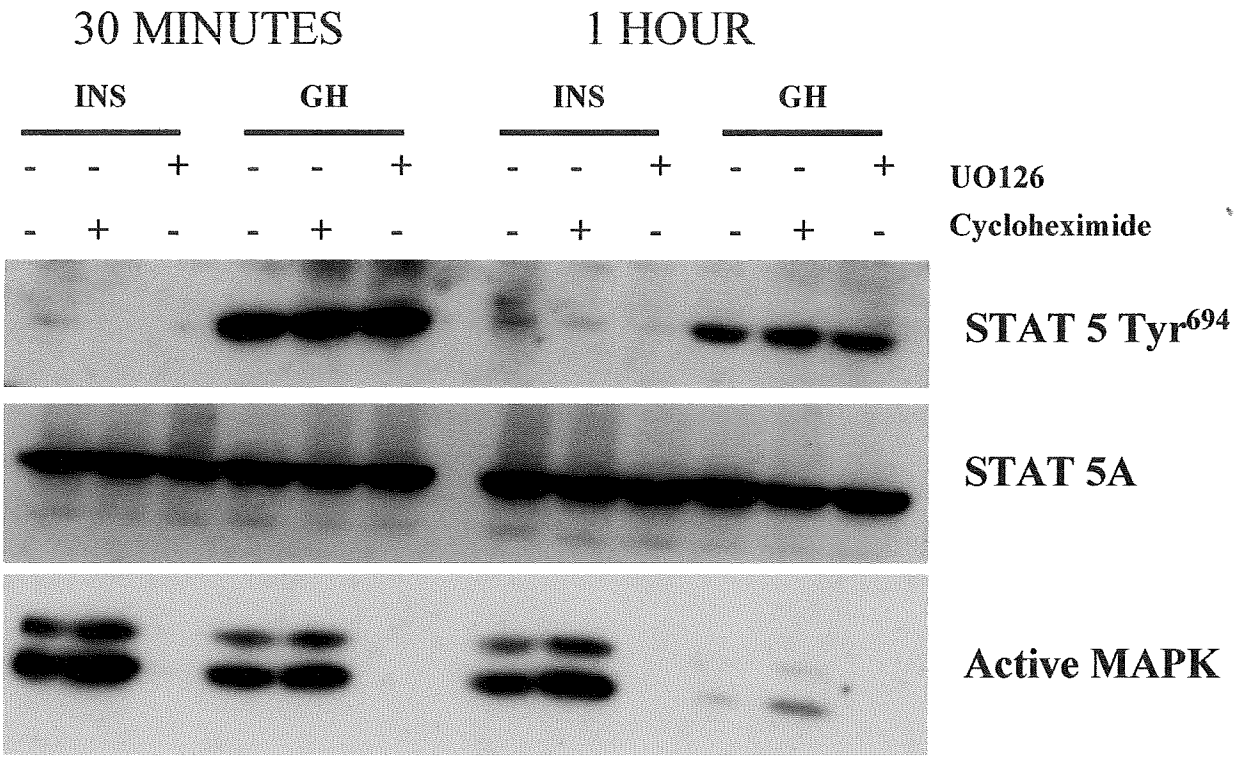

INS
GH
0 .5 1 3 6 8 0 .5 1 3 6 8
Hours
SOCS-3 mRNA

Figure 2

**Figure 3. The time dependent modulation of SOCS-3 mRNA by GH or INS in the presence of a MEK inhibitor or cycloheximide.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated with INS or GH for the times indicated. For each time point, some cells were pretreated for 40 minutes with either 5 mM cycloheximide or 5 mM of the MEK inhibitor, U0126. A. Whole cell extracts were isolated from fully differentiated 3T3-L1 adipocytes that were treated with either growth hormone (GH) or insulin (INS) for the times indicated at the top of the figure. One hundred mg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. Samples were processed and results were visualized as described in Figure Legend 1. B. Total RNA was isolated after GH or INS treatment. Fifteen mg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern Blot Analysis. This is a representative experiment independently performed two times.

Figure 3

## A. Western




## B. Northern

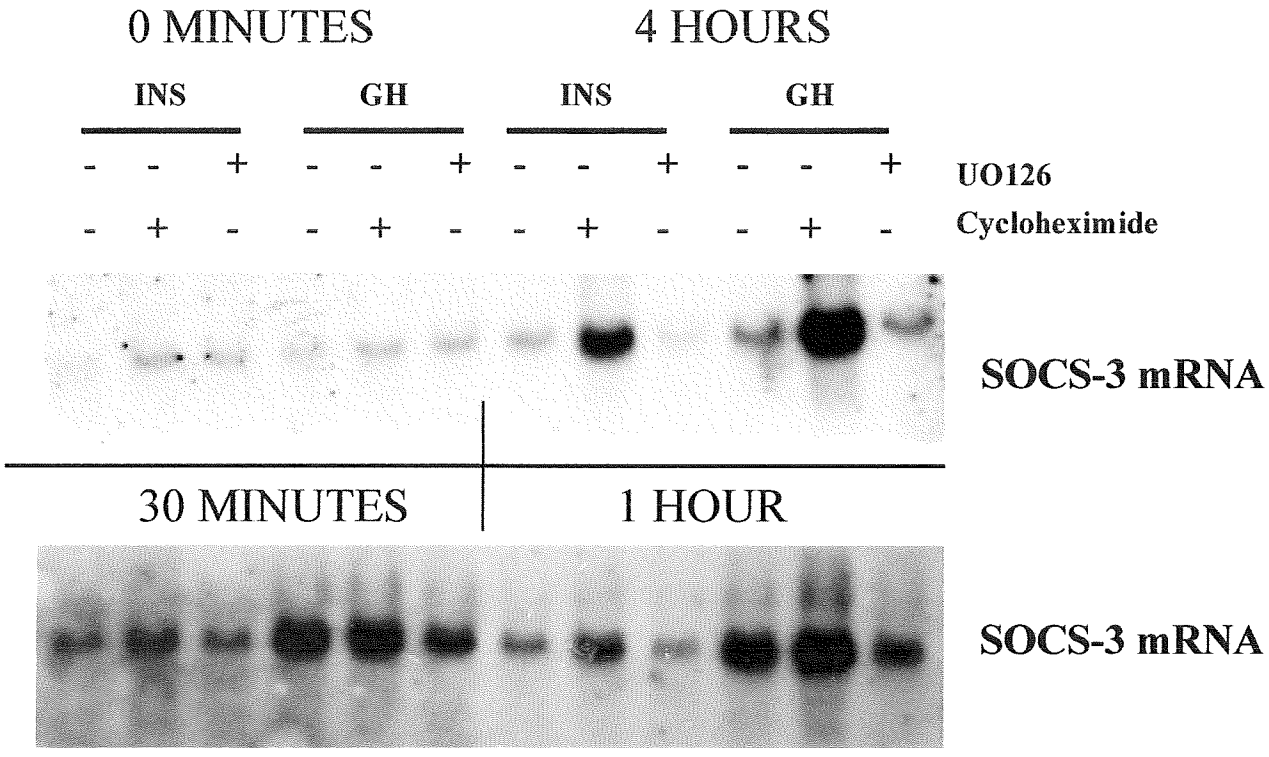


**Figure 4. The GH induction of SOCS-3 mRNA is unaffected by the inhibition of MAPK activity and enhanced in the presence of cycloheximide.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated with GH for the times indicated. Following a 40 minute pretreatment with either 5 mM cycloheximide (CH) or 5 mM of the MEK inhibitor (U0126) cells were harvested at various time points indicated in the figure. One hundred mg of each whole cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. To examine SOCS-3 mRNA, fifteen mg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern Blot Analysis. This is a representative experiment independently performed three times.

Figure 4

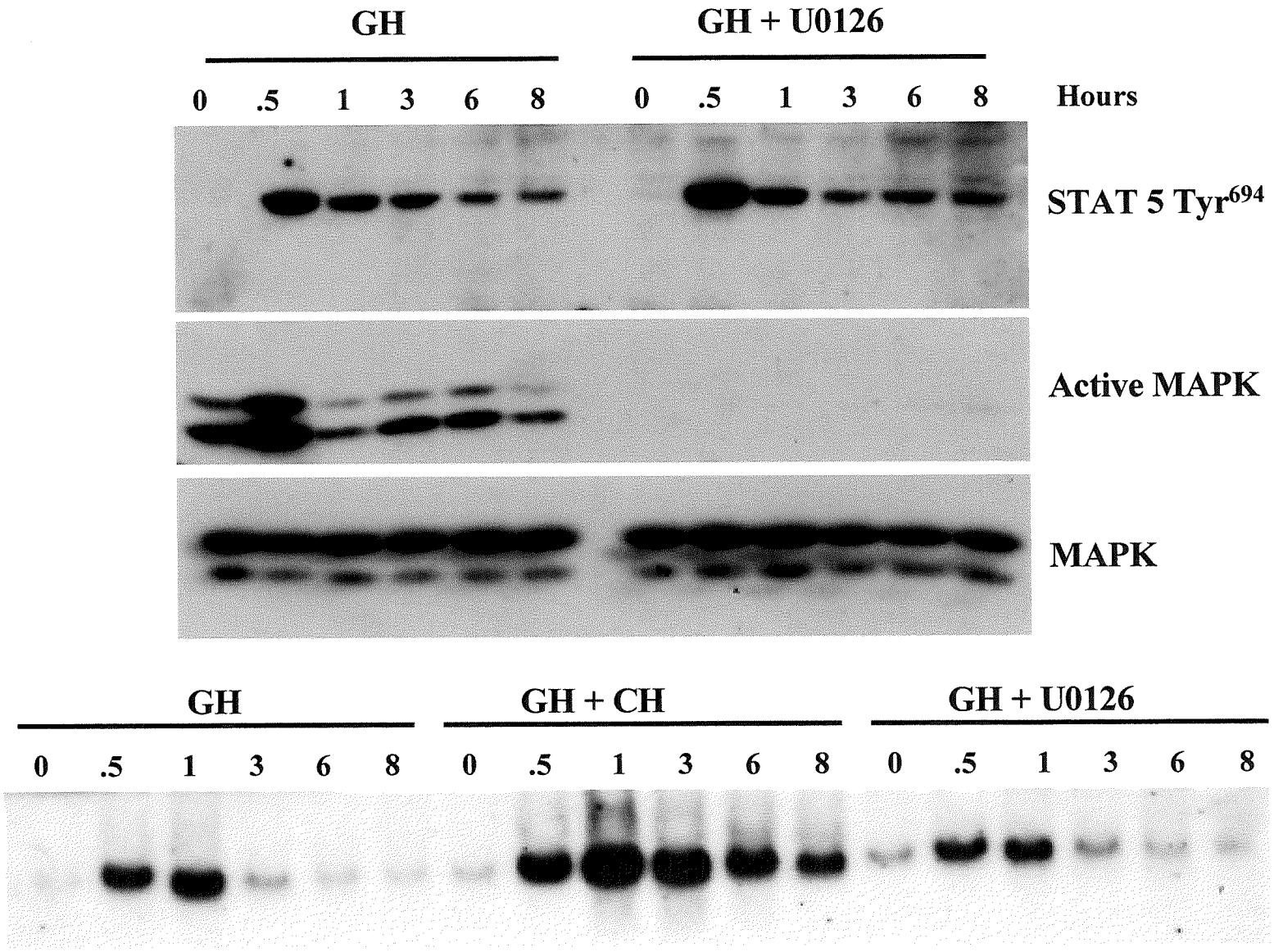

GH
GH + U0126
0 .5 1 3 6 8
0 .5 1 3 6 8
Hours
STAT 5 Tyr694
Active MAPK
MAPK
GH
GH + CH
GH + U0126
0 .5 1 3 6 8
0 .5 1 3 6 8
0 .5 1 3 6 8
Hours
SOCS-3
mRNA

**Figure 5. The ability of growth hormone pretreatment to inhibit acute GH induced STAT 5 phosphorylation is independent of new protein synthesis or active MAPK.** A. Fully differentiated 3T3-L1 adipocytes were treated with for 15 min. with growth hormone in the presence or absences of a GH pretreatment for 2, 8, or 18 hours. B. Fully differentiated 3T3-L1 adipocytes were treated with for 15 min. with growth hormone following a 3 hr. GH pretreatment in the presence or absence of a 5 mM cycloheximide (CH). C. Fully differentiated 3T3-L1 adipocytes were treated with for 15 min. with growth hormone following a 3 hr. GH pretreatment in the presence or absence of a 5 mM of U0126 (U2). For each part of the figure, whole cell extracts was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. This is a representative experiment independently performed three times.

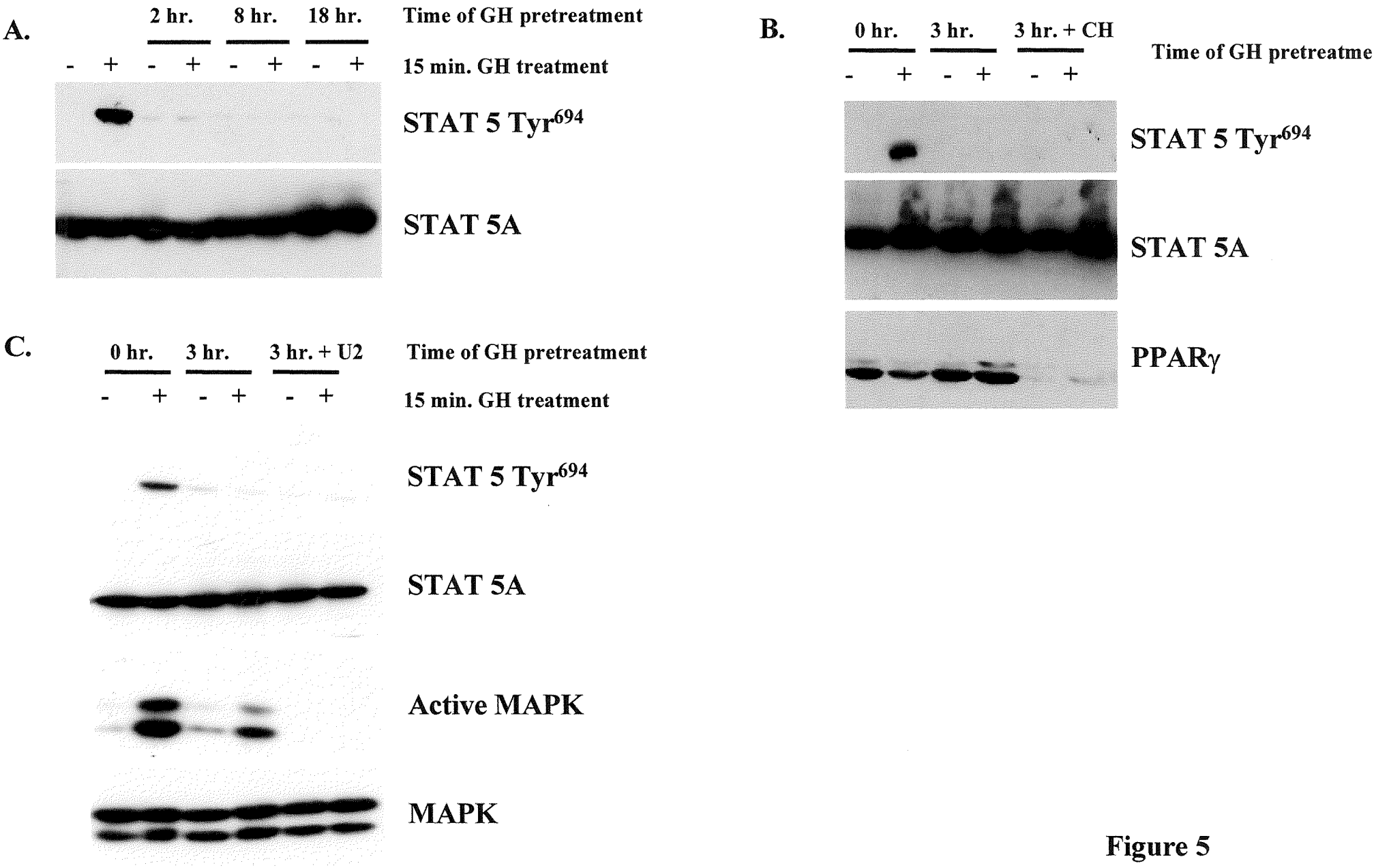

A.
2 hr.
8 hr.
18 hr.
Time of GH pretreatment
- + - + - + - +
15 min. GH treatment
STAT 5 Tyr694
STAT 5A
B.
0 hr.
3 hr.
3 hr. + CH
Time of GH pretreatme
- + - + - +
STAT 5 Tyr694
STAT 5A
PPARγ
C.
0 hr.
3 hr.
3 hr. + U2
Time of GH pretreatment
- + - + - +
15 min. GH treatment
STAT 5 Tyr694
STAT 5A
Active MAPK
MAPK

Figure 5

**Figure 6. Cycloheximide induces SOCS-3 mRNA in the presence and absence of GH.** Whole cell extracts and total RNA was isolated from fully differentiated 3T3-L1 adipocytes that were treated with GH for the times indicated in the presence and absence of 5 mM cycloheximide (CH). One hundred mg of each whole cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. To examine SOCS-3 mRNA, fifteen mg of total RNA was electrophoresed, transferred to nylon, and subjected to Northern Blot Analysis. This is a representative experiment independently performed two times.

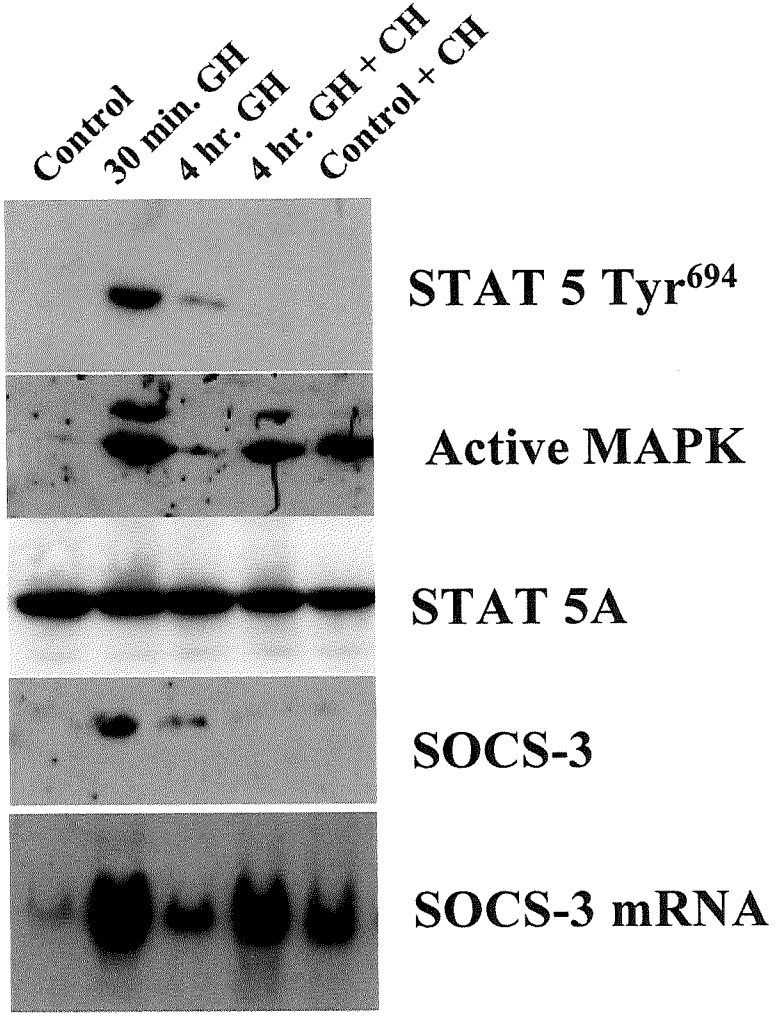

Control
30 min. GH
4 hr. GH
4 hr. GH + CH
Control + CH
STAT 5 Tyr694
Active MAPK
STAT 5A
SOCS-3
SOCS-3 mRNA

Figure 6

**Figure 7. GH induction of STAT 5 activation is inhibited by GH pretreatment, but not by insulin pretreatment**. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes A. Cells were untreated (-) or treated for 15 minutes with Insulin (I) or GH (+). Some cells were pretreated with insulin or GH for 3 hours and 18 hours. B. Cells were untreated (-) or treated for 15 minutes with GH (G) or Insulin (+). In addition, some cells were pretreated with insulin or GH for 3 hours and 18 hours. C. Cells were acutely treated with GH (G) or insulin (I) or untreated (-) after 3 hour and 18 hour pretreatments as indicated at the top of the figure. For each panel, one hundred mg of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to western blot analysis. This is a representative experiment independently performed four times.

## A. Acute GH treatment -/+ GH or INS pretreatment

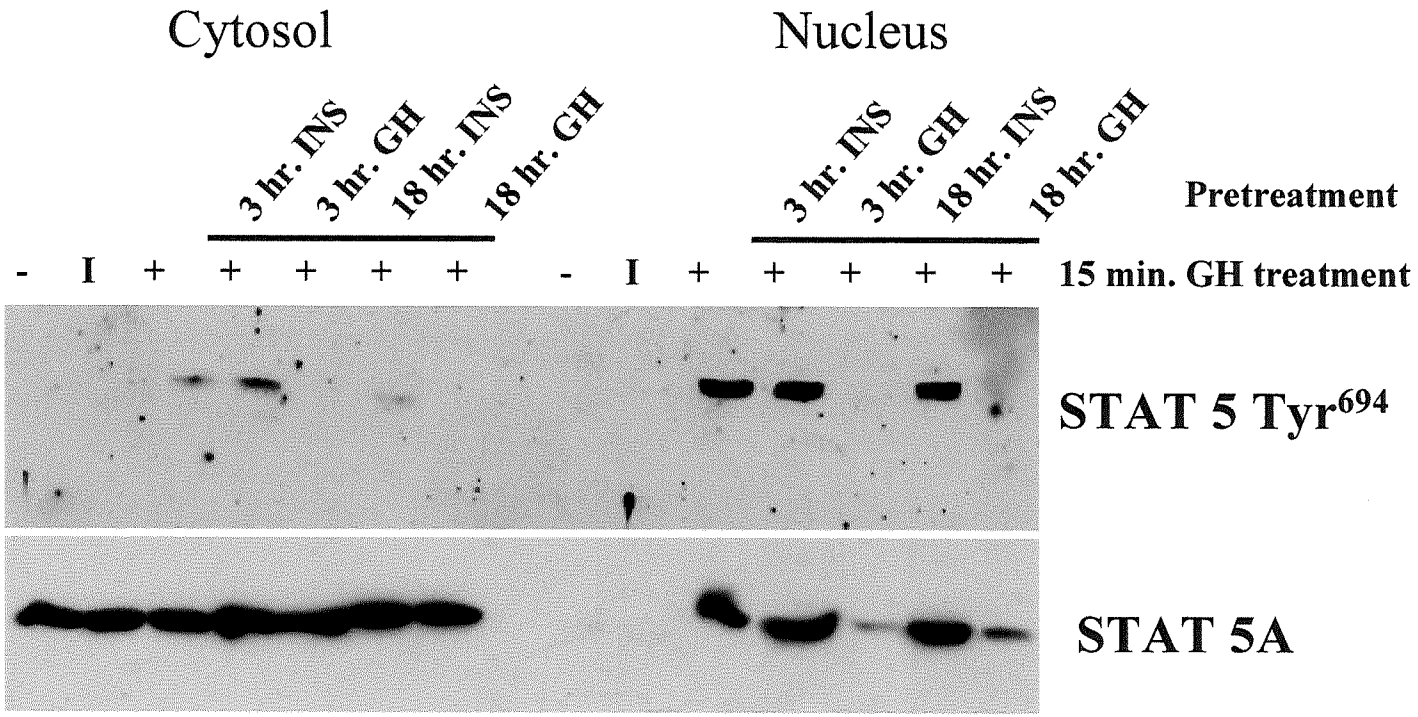



Figure 7A

## B. Acute INS treatment -/+ GH or INS pretreatment

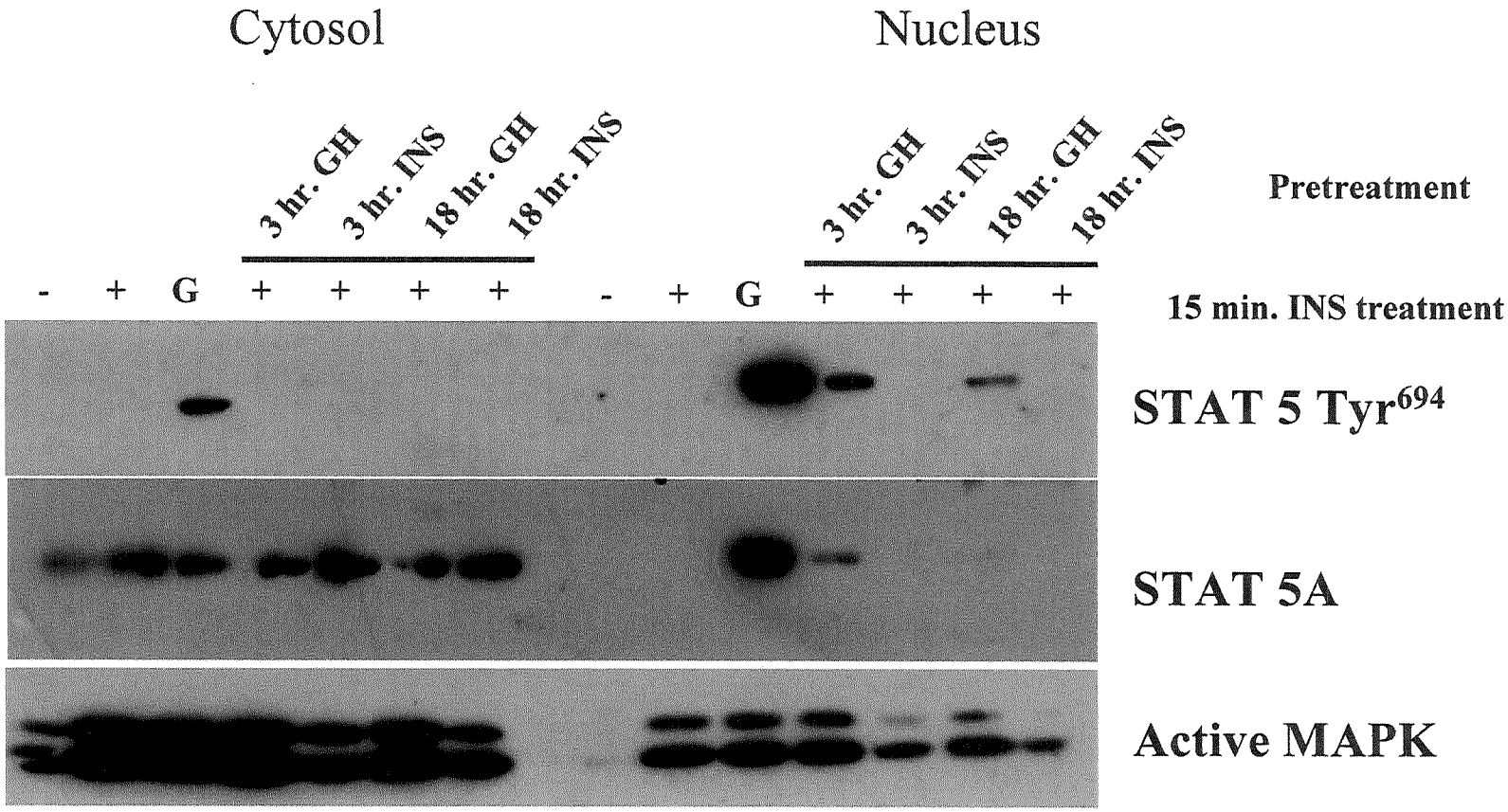



Figure 7B

## C. Acute treatments -/+ GH or INS pretreatment

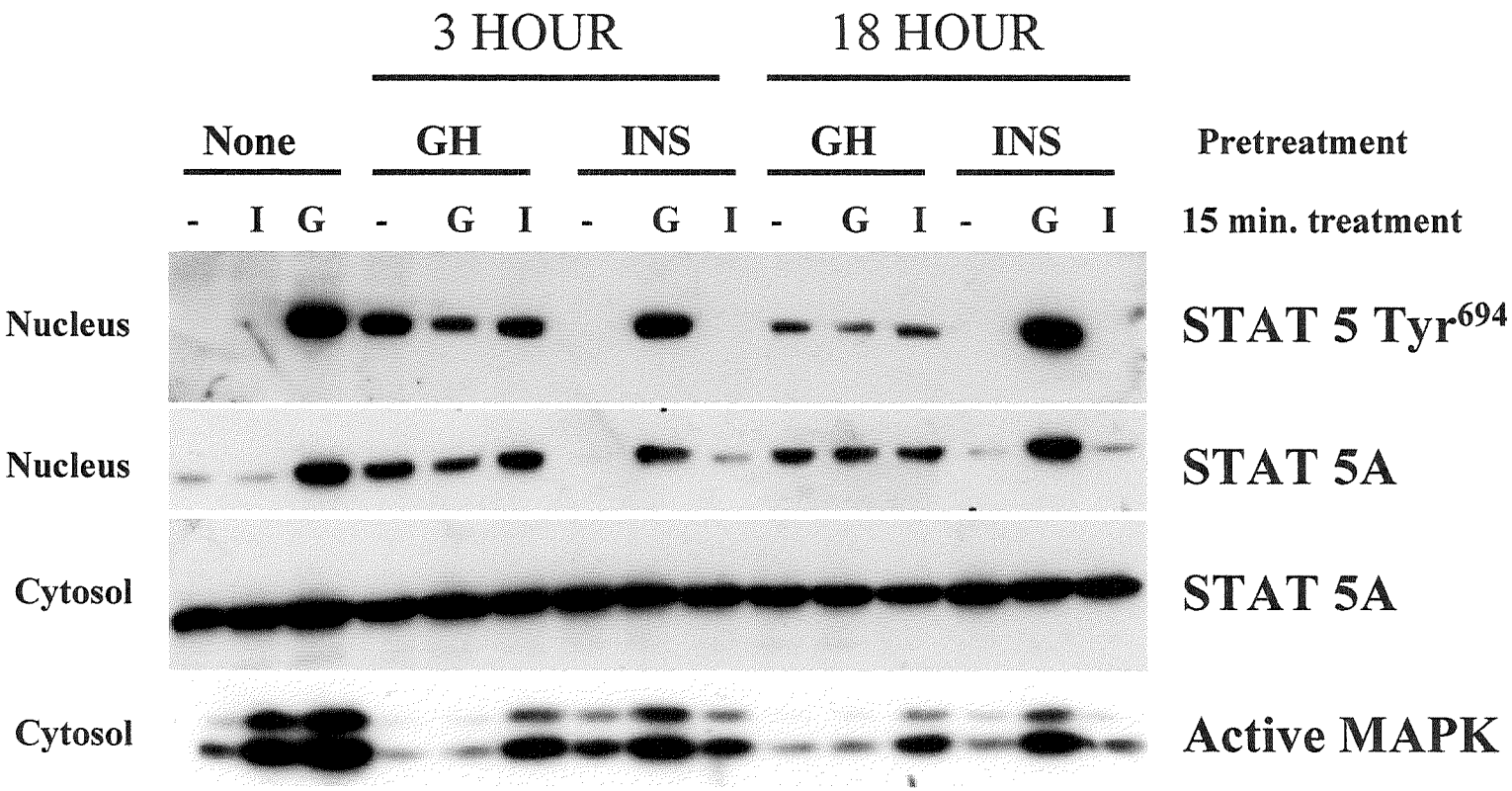



Figure 7C

## REFERENCES


1 http://www.cdc.gov/nchs/fastats/lcod.htm

2 Quinn, L. (2001) *Nurs. Clin. North Am.* **36**(2): 175-192

3 Eschwege, E. (2000) *Arch. Mal. Couer. Vaiss.* **93**(4): 13-17

4 Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.B., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. (2001) *Nature* **409**: 301-312

5 Hensrud, D.D. (2001) *Obes. Res.* **9**(4): 348S-353S

6 Greenway, F. (1999) *Diabetes Technol. Ther.* **1**(3): 277-288

7 Taha, C., and Klip, A. (1999) *J. Membrane Biol.* **169**: 1-12

8 Balhoff, J.P., and Stephens, J.M. (1998) *Biochem. Biophys. Res. Commun.* **247**(3): 894-900

9 Gerland, K., Bataille-Simoneau, N., Basle, M., Fourcin, M., Gascan, H., and Mercier, L. (2000) *Mol. Cell Endocrinol.* **158**(1-2): 1-9

10 Gregory, S.H., Wing, E.J., Danowski, K.L., van Rooijen, N., Dyer, K.F., and Tweardy, D.J. (1998) *J. Immunol.* **160**(12): 6056-6061

11 Halfter, H., Friedrich, M., Postert, C., Ringelstein, E.B., and Stogbauer, F. (1999) *Mol. Cell Biol. Res. Commun.* **1**(2): 109-116

12 Kelly, P.A., Finidori, J., Moulin, S., Kedzia, C., and Binart, N. (2001) *Horm. Res.* **55**(2): 14-17

13 Yamaoka, K., Otsuka, T., Niiro, H., Nakashima, H., Tanaka, Y., Nagano, S., Ogami, E., Niho, Y., Hamasaki, N., and Izuhara, K. (1999) *J. Interferon Cytokine Res.* **19**(6): 679-685

14 Adams, T.E., Hansen, J.A., Starr, R., Nicola, N.A., Hilton, D.J., and Billestrup, N. (1998) *J. Biol. Chem.* **273**(3): 1285-1287

15 Bousquet, C., Susini, C., and Melmed, S. (1999) *J. Clin. Invest.* **104**(9): 1277-1285

16 Magrangeas, F., Boisteau, O., Denis, S., Jacques, Y., and Minvielle, S. (2001) *Eur. Cytokine Netw.* **12**(2): 309-315

17 Sakamoto, H., Yasukawa, H., Masuhara, M., Tanimura, S., Sasaki, A., Yuge, K., Ohtsubo, M., Ohtsuka, A., Fujita, T., Ohta, T., Furukawa, Y., Iwase, S., Yamada, H., and Yoshimura, A. (1998) *Blood* **92**(5): 1668-1676

18 Sporri, B., Kovanen, P.E., Sasaki, A., Yoshimura, A., and Leonard, W.J. (2001) *Blood* **97**(1): 221-226

19 Sawka-verhelle D., Filloux, C., Tartare-Deckert, S., Mothe, I., and Van Obberghen, E. (1997) *Eur. J. Biochem.* **250**(2): 411-417

20 Chen, J., Sadowski, H.B., Kohanski, R.A., and Wang, L.H. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**(6): 2295-2300

21 Storz, P., Doppler, H., Pfizenmaier, K., and Muller, G. (1999) *FEBS Lett.* **464**(3): 159-163

22 Sawka-verhelle D., Tartare-Deckert, S., Decaux, J.F., Girard, J., and Van Obberghen, E. (2000) *Endocrinology* **141**(6): 1977-1988

23 Emanuelli, B., Peraldi, P., Filloux, C., Sawka-Verhelle, D., Hilton, D., and Van Obberghen, E. (2000) *J. Biol. Chem.* **275**(21): 15985-15991

24 Peraldi, P., Filloux, C., Emanuelli, B., Hilton, D.J., and Van Obberghen, E. (2001) *J. Biol. Chem.* **276**(27): 24614-24620

25 Mooney, R.A., Senn, J., Camerson, S., Inamdar, N., Boivin, L.M., Shang, Y., and Furlanetto, R.W. (2001) *J. Biol. Chem.* **276**(28): 25889-25893

26 Zhang, J.G., Metcalf, D., Rakar, S., Asimakis, M., Greenhalgh, C.J., Willson, T.A., Starr, R., Nicholson, S.E., Carter, W., Alexander, W.S., Hilton, D.J., and Nicola, N.A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**(23): 13261-13265

27 Krebs, D.L., and Hilton, D.J. (2001) *Stem Cells* **19**(5): 378-387

28 Marine, J.C., McKay, C., Wang, D., Topham, D.J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., and Ihle, J.N. (1999) *Cell* **98**(5): 617-627

29 Metcalf, D. (1999) *Exp. Hematol.* **27**(12): 1715-1723

30 Gisselbrecht, S. (1999) *Eur. Cytokine Netw.* **10**(4): 463-470

31 Nicola, N.A., and Greenhalgh, C.J. (2000) *Exp. Hematol.* **28**(10): 1105-1112

32 Kile, B.T., and Alexander, W.S. (2001) *Cell Mol. Life Sci.* **58**(11): 1627-1635

33 Finidori, J. (2000) *Vitam. Horm.* **59**: 71-97

34 Laemlli, U.K., Beguin, F., and Gujer-Kellenberger, G. (1970) *J. Mol. Biol.* **47**(1): 69-85

35 Lee, J.K., Won, J.S., Choi, M.R., Kim, Y.H., and Suh, H.W. (2001) *Mol. Cells* **12**(1): 11-16

36 Morawietz, H., Ma, Y.H., Vives, F., Wilson, E., Sukhatme, V.P., Holtz, J., and Ives, H.E. (1999) *Circ. Res.* **84**(6): 678-687

37 Roger, T., Out, T.A., Jansen, H.M., and Lutter, R. (1998) *Biochim. Biophys. Acta* **1398**(3): 275-284

38 Emmanuelli, B., Peraldi, P., Filloux, C., Chavey, C., Freidinger, K., Hilton, D.J., Hotamiligil, G.S., and Van Obberghen, E. (2001) *J. Biol. Chem.* **276**(51): 47944-47949