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MODULATION OF ADIPOCYTE GENES BY SIGNAL TRANSDUCERS AND
ACTIVATORS OF TRANSCRIPTION

A Dissertation

Submitted to the Graduate Faculty of the Louisiana
State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by Jessica C. Hogan
B.S., Southeast Missouri State University, 1997
M.S., Middle Tennessee State University, 2000
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
ABSTRACT.....	ix
CHAPTER 1: INTRODUCTION.....	1
1.1 Obesity and Adipocytes.....	1
1.2 Adipocyte Proteins.....	7
1.3 JAK/STAT Signaling.....	13
1.4 Cytokines.....	19
1.5 Rationale.....	22
CHAPTER 2: EXPERIMENTAL PROCEDURES.....	24
2.1 Materials.....	24
2.2 Cell Culture.....	24
2.3 Preparation of Whole Cell Extracts.....	25
2.4 Preparation of Nuclear and Cytosolic Extracts.....	25
2.5 Gel Electrophoresis.....	26
2.6 RNA Analysis.....	26
2.7 Electrophoretic Mobility Shift Assay.....	26
2.8 Constructs.....	27
2.9 Transient Transfections.....	28
2.10 Determination of [H^3]2-Deoxyglucose Uptake.....	28
CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF A STAT1 BINDING SITE IN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ 2 PROMOTER.....	29
3.1 Introduction.....	29
3.2 Results.....	30
3.3 Discussion.....	39
CHAPTER 4: STAT1 BINDS TO THE LIPOPPROTEIN LIPASE PROMOTER <i>IN VITRO</i>	44
4.1 Introduction.....	44
4.2 Results.....	44
4.3 Discussion.....	48
CHAPTER 5: REGULATION OF FATTY ACID SYNTHASE BY STAT5.....	51
5.1 Introduction.....	51
5.2 Results.....	51
5.3 Discussion.....	58

CHAPTER 6: THE EFFECTS OF LEUKEMIA INHIBITORY FACTOR ON ADIPOCYTES.	63
6.1 Introduction.....	63
6.2 Results.....	64
6.3 Discussion.....	71
CHAPTER 7: SUMMARY AND CONCLUSIONS.....	77
LITERATURE CITED.....	81
APPENDIX: PERMISSION TO REPRINT.....	101
VITA.....	104

LIST OF TABLES

Table 3.1 Potential STAT1 binding sites in the PPAR γ 2 promoter.....	30
Table 4.1 STAT1 binding sites in the LPL promoter.....	45
Table 5.1 Potential STAT binding sites in the FAS promoter.....	56
Table 6.1 STAT binding sites in the C/EBP δ promoter.....	70

LIST OF FIGURES

3.1	The identification of an IFN γ -sensitive element in the PPAR γ 2 promoter.....	31
3.2	Binding to the -221 to -207 site of the PPAR γ 2 promoter is specific.....	32
3.3	STAT1 binds to the PPAR γ 2 promoter <i>in vitro</i>	33
3.4	IFN γ modulates the activity of the PPAR γ 2 promoter.....	35
3.5	LIF-activated STAT1 binds to the PPAR γ 2 promoter.....	37
3.6	Acute LIF treatment does not affect the levels of PPAR γ mRNA in adipocytes.....	38
3.7	CT-1 induces STAT1 binding to the -221 to -207 site of the PPAR γ 2 promoter <i>in vitro</i>	40
4.1	The identification of a specific IFN γ -sensitive binding site in the LPL promoter.....	46
4.2	IFN γ -induced STAT1 binds to the LPL promoter <i>in vitro</i>	47
4.3	IFN γ treatment decreases LPL protein levels.....	48
5.1	Activators of STAT5 decrease expression of FAS in 3T3-L1 adipocytes.....	52
5.2	PRL does not affect turnover of FAS protein.....	53
5.3	A PRL-responsive region resides between -1594 to -700 of the rat FAS promoter.....	55
5.4	PRL stimulates binding of nuclear proteins to the -908 to -893 site in the rat FAS promoter.....	57
5.5	PRL induces STAT5A binding to the -908 to -893 site in the rat FAS promoter.....	58
5.6	The -908 to -893 region of the rat FAS promoter confers sensitivity to PRL.....	59
6.1	LIF does not affect the differentiation of 3T3-L1 adipocytes.....	65
6.2	Acute LIF treatment does not affect expression of adipocyte marker proteins.....	66
6.3	The effects of chronic treatment of LIF on 3T3-L1 adipocytes.....	67
6.4	LIF does not affect insulin-stimulated glucose uptake in 3T3-L1 adipocytes.....	68
6.5	LIF induces the expression of C/EBP δ and SOCS3 mRNA.....	69

6.6	LIF activates binding by protein complexes from the nuclei of 3T3-L1 adipocytes to three sites of the C/EBP δ promoter.....	70
6.7	Binding of LIF-activated STAT1, not STAT3, to the three sites in the C/EBP δ promoter...	72

ABSTRACT

Members of the STAT transcription factor family are expressed in adipocytes, including STATs 1, 3, 5A, 5B, and 6. Although STATs 1 and 5 proteins are known to be induced during adipogenesis, the functions of these STATs in mature adipocytes are not known. Hence we have sought to identify adipocyte genes which are transcriptionally regulated by STATs to elucidate a role of these proteins in fat cells. We have characterized STAT binding sites in the promoters of four adipocyte genes, PPAR γ 2, LPL, FAS and C/EBP δ . PPAR γ 2 expression decreases in adipocytes following exposure to IFN γ , an activator of STAT1. IFN γ induces the binding of STAT1 to a site in the PPAR γ 2 promoter. Furthermore, the STAT1 binding site is required for IFN γ regulation of the PPAR γ 2 promoter *in vitro*. Although both CT-1 and LIF induced STAT1 binding to the PPAR γ 2 promoter, only CT-1 substantially modulated expression of PPAR γ 2. We have also identified a STAT1 binding site in the promoter of LPL, which is bound by STAT1 following treatment of 3T3-L1 adipocytes with IFN γ . In addition, LPL protein levels are downregulated by IFN γ treatment. Treatment with PRL, an activator of STAT5A, decreased levels of FAS protein and mRNA in adipocytes. Regulation of the rat FAS promoter by PRL required a region of the promoter which contained a STAT5 binding site. Binding to this site by STAT5A was activated by PRL treatment and was highly specific. Finally, in our analysis on the effects of LIF on adipocytes, we determined that the expression SOCS3 and C/EBP δ mRNA transiently increases following treatment of adipocytes with LIF. We identified three STAT1 binding sites within the C/EBP δ promoter, which we hypothesize mediate the induction of C/EBP δ by LIF. Although LIF did not profoundly affect adipogenesis or basal and insulin-stimulated glucose uptake, chronic treatment with LIF abrogated the level of SREBP1 and FAS proteins. In summary, our studies suggest that STAT1 and STAT5 serve to limit synthesis of

lipids in mature fat cells, limiting expansion of adipocytes and accretion of adipose tissue. The identification of PPAR γ , FAS, and LPL as STAT-regulated genes provides insight into the molecular mechanisms of energy homeostasis, adipocyte physiology and the action of cytokines in fat.

CHAPTER 1: INTRODUCTION

1.1 Obesity and Adipocytes

During the past two decades obesity has become an epidemic. The prevalence of obesity is rapidly on the rise not only in industrialized nations, but also in countries with developing economies (Speakman, 2004). In the United States of America, more than 50% of the population is overweight and nearly 20% are obese (Speakman, 2004). Recent heightened awareness and interest in obesity has lead to the development of public health strategies that promote healthy eating and physical fitness and has made evident the importance of research in the field of obesity.

Industrialization is correlated with population trends of increased food intake and decreased physical activity, the so-called Western lifestyle (reviewed in French *et al.*, 2001). In the last 30 years, overall availability of energy from food per person in the United States has increased 15%. Furthermore, in the same period of time, consumption of milk has declined by over 20%, while soft drink consumption has more than doubled, concurrent with the increased intake of sugar. Moreover, the frequency of eating away from home, particularly at fast food restaurants has increased, where increased portion sizes and high density of calories and fat result in underestimation of actual food consumption. Energy expenditure in the American population has declined as television viewing has replaced more physically engaging leisure activities and as car ownership has become widespread. In addition, labor-saving technological advances have decreased the physical activity of household chores and workplace tasks. In the last century, service and high technology careers have become the more prevalent types of work, a shift from the heavy manual labor of farming, carpentry, and factory work. Clearly, there are multiple environmental causes of the obesity epidemic in societies of affluence.

Obesity is most commonly defined by body mass index (BMI), calculated as weight in kilograms divided by height in meters squared (Speakman, 2004). A BMI between 18.5 and 24.9 is considered normal, the BMI of overweight individuals is between 25 and 29.9, and over 30.0 is considered obese (Speakman, 2004). BMI is a reliable measure of body composition only if body fatness varies with body weight but is not a good indicator for athletes with increased muscle mass or the elderly who may have disproportionately less lean mass (Speakman, 2004; Elia, 2001). Other measures of body fat include ratio of waist and hip circumferences, underwater weighing, skin fold measurement, and electrical impedance (Bray, 2001).

As the prevalence of obesity increases, the health care costs of obesity are rising as well and are estimated to be 7% of the total health costs in the United States (Colditz, 1999). The costs associated with obesity are mainly not due to obesity itself, but instead, are attributable to several obesity-related diseases: diabetes, heart disease, hypertension, stroke, gall bladder disease, renal disease, cancer, liver disease, and osteoarthritis (Speakman, 2004). The risks of these diseases decrease with even moderate weight loss (Visscher and Seidell, 2001). Obesity is associated with a tenfold increase in risk of type 2 diabetes, the most expensive consequence of obesity (Carey *et al.*, 1997).

Diabetes is a disease characterized by increased levels of blood glucose. Type 1 diabetes or juvenile diabetes, is an autoimmune disease in which the insulin-producing pancreatic beta cells are destroyed (Dean and McEntyre, 2004). Over 16 million Americans have type 2 diabetes or noninsulin dependent diabetes mellitus (NIDDM), which is generally associated with insulin resistance, resulting in decreased uptake of glucose by muscle and adipose tissue and a failure to suppress glucose production in the liver (Dean and McEntyre, 2004). Increased levels of free fatty acids in circulation are associated with excess adiposity (Boden, 1997). Free fatty

acids impair insulin action at multiple levels, decreasing glucose uptake and oxidation in insulin-sensitive tissues (Boden, 1997), attenuating the inhibitory effect of insulin on hepatic glucose output (Boden, 1997), and inducing apoptosis in pancreatic β cells, resulting in decreased insulin secretion (Cnop *et al.*, 2001). Lipotoxicity may not be the only molecular link between obesity and diabetes, as multiple adipocyte-derived messengers have been identified that affect insulin sensitivity.

Obesity results from both quantitative and qualitative changes in adipose tissue. Adipocytes have the unique capacity to store substantial volumes of lipid in the form of triglycerides as energy intake exceeds energy needs and to mobilize lipids as free fatty acids to supply the energy needs of other tissues (Rosen and Spiegelman, 2000). Adipocytes expand to the maximum volume, and then the tissue increases the number of cells to accommodate the storage needs (Rosen and Spiegelman, 2000). While the major function of white adipose tissue (WAT) is storage, brown adipose tissue (BAT) dissipates energy as heat through the uncoupling of respiration from the synthesis of ATP which is conferred by increased number of mitochondria and expression of uncoupling protein-1 (Nedergaard *et al.*, 1986). BAT may also function to protect against obesity. Depots of brown adipocytes are highly diminished in adult humans, but are more prominent in mature rodents (Nedergaard *et al.*, 1986).

The concept of the adipocyte as a dynamic component of energy homeostasis was proposed many years ago (Kennedy, 1953), but only recently has there been evidence to support this hypothesis through the identification of secreted molecules from adipocytes. These include hormones: leptin and resistin; cytokines: tumor necrosis factor α (TNF α) and interleukin 6 (IL-6); peptides: plasminogen activator inhibitor-1 and angiotensinogen; and adipokines: adiponectin and adipisin; in addition to free fatty acids and glycerol (Frühbeck *et al.*, 2001). These products

of adipocytes have autocrine, paracrine, and endocrine effects, forming signaling circuits with the hypothalamus, pancreas, liver, skeletal muscle, kidneys, endothelium, and immune system to regulate energy homeostasis, insulin sensitivity, satiety, and vascular function (Frühbeck *et al.*, 2001). These factors also exhibit altered levels in the condition of obesity, fasting, and refeeding, indicative of their importance in metabolism (Rosen and Spiegelman, 2000). In addition, adipocytes are responsive to many signals originating from other tissues (Frühbeck *et al.*, 2001).

Although a previous hypothesis suggested that people are born with all of the adipocytes they would need, there is substantial evidence that adipogenesis occurs throughout the life of an organism (Prins and O’Rahilly, 1997). New adipocytes arise due to normal cell turnover and to meet requirements for increased energy storage (Prins and O’Rahilly, 1997). *In vivo*, the development of white adipose tissue (WAT) begins just prior to birth, with a rapid expansion following birth (Gregoire *et al.*, 1998). The early events of adipogenesis, committing mesodermal precursor cells to preadipocytes are not well understood; however, the proliferation of a network of capillaries in a region of subcutaneous loose connective tissue is considered primitive adipose tissue (Rosen and Spiegelman, 2000). Several molecular events and transcriptional pathways that govern differentiation of preadipocytes to adipocytes have been identified. These discoveries were possible largely due to the establishment of the 3T3-L1 and 3T3-F442A preadipocyte cell lines from the Swiss 3T3 cells derived from 17- to 19-day mouse embryos (Green and Meuth, 1974; Green and Kehinde, 1975; 1976). Preadipocytes are morphologically indistinguishable from fibroblasts; yet they are committed to the adipocyte lineage (Rosen and Spiegelman, 2000). The first stage of differentiation is growth arrest, which is achieved in culture by contact inhibition and by the addition of a differentiation promoting

cocktail of hormones, methylisobutylxanthine (MIX), dexamethasone, and insulin (Green and Kehinde, 1975). The effects of insulin on preadipocytes are not mediated by the insulin receptor, but instead occur through activation of the insulin-like growth factor 1 receptor (IGF-1 R). This cross-activation by insulin occurs with a super physiological dose of insulin (Rosen and Spiegelman, 2000). Stimulation of the IGF-1 R activates the Ras/mitogen activated protein kinase (MAPK) pathway and Akt (Rosen and Spiegelman, 2000). Constitutively active Akt has been shown to induce spontaneous adipogenesis in 3T3-L1 cells, indicating that Akt may be the critical signaling cascade activated by the IGF-1 R (Magun *et al.*, 1996). MIX is a phosphodiesterase inhibitor which increases levels of cAMP (Rosen and Spiegelman, 2000). Although MIX has other effects, the predominant effector of differentiation is likely cAMP (Yarwood *et al.*, 1995). Dexamethasone is a synthetic glucocorticoid activating the glucocorticoid receptor (GR). There is evidence to indicate that dexamethasone inhibits the expression of preadipocyte factor-1 (Pref-1), a protein that has anti-adipogenic properties (Smas *et al.*, 1999). These three agents accelerate adipogenesis under serum-free conditions, but their effects on adipogenesis are potentially effective in the presence of fetal calf serum. One component of fetal calf serum that appears to mediate the proadipogenic effects is growth hormone (GH), which has been shown to induce differentiation in multiple cell lines (Catalioto *et al.*, 1992; Tominaga *et al.*, 2002; Stewart *et al.*, 2004). Yet, GH inhibits differentiation of primary preadipocyte cultures (Hausman and Martin, 1989; Wabitsch *et al.*, 1996; Hansen *et al.*, 1998), indicating that the effects of GH depend on the stage of differentiation.

Following growth arrest, preadipocytes undergo clonal expansion followed by a second phase of growth arrest which is associated with changes in expression of several proteins involved in cell cycle control, p18, p21, and p27 (Morrison and Farmer, 1999) and the initiation

of a transcriptional cascade. CCAAT/enhancer binding protein- δ (C/EBP δ) and C/EBP β are transiently induced by dexamethasone and MIX, respectively (Cao *et al.*, 1991; Wu *et al.*, 1996), which is followed by the expression of two transcription factors that are highly adipogenic, peroxisome proliferator-activated receptor- γ (PPAR γ) and C/EBP α . PPAR γ induces growth arrest through upregulation of p18 and p21 (Rosen and Spiegelman, 2000) and has been shown to activate the expression of several adipocyte marker proteins, such as fatty acid binding protein aP2 and phosphoenolpyruvate carboxykinase (Tontonoz *et al.*, 1994; 1995). Following the second phase of growth arrest, cells acquire a round morphology, accumulate lipid, become insulin sensitive (MacDougald and Lane, 1995) and express genes that are associated with the adipocyte phenotype, aP2, glycerophosphate dehydrogenase, lipoprotein lipase (LPL), fatty acid synthase (FAS), signal transducer and activator of transcription 5 (STAT5), glucose transporter 4 (GLUT4), and the insulin receptor (IR) (Spiegelman *et al.*, 1993; Stephens *et al.*, 1996).

The cell lines, 3T3-L1 and 3T3-F442A, in addition to other adipocyte cell models, exhibit many features of *in vivo* adipocytes, but they do not perfectly recapitulate adipose tissue. Cell lines synthesize and secrete relatively low levels of leptin and TNF α in comparison to *in vivo* adipose tissue (Rosen and Spiegelman, 2000). Furthermore, cell lines do not exhibit the depot-specific characteristics that have been demonstrated for perigonadal, omental, retroperitoneal and subcutaneous fat depots (Ostman *et al.*, 1979; Djian *et al.*, 1985; Adams *et al.*, 1997; Morimoto *et al.*, 1997; Lefebvre *et al.*, 1998). Moreover, the use of cell lines does not include the inputs from the matrix of connective tissue, neurons or stromovascular cells that are part of the adipose tissue (Rosen and Spiegelman, 2000). In spite of these caveats, studies of adipocyte function using cell lines are extremely useful and have yielded valuable insights into adipocyte biology. Cell lines are a uniform population of cells which simultaneously differentiate in culture, unlike

the diffuse adipocyte precursors in adipose tissue and heterogeneous primary cultures which cannot be synchronized (Ntambi and Young-Cheul, 2000). Moreover, primary cultures require a large amount of fat tissue and have a limited lifespan in culture, unlike cell lines which may be passaged many times (Ntambi and Young-Cheul, 2000). Therefore, the 3T3-L1 cells, in addition to other adipocyte cell lines, are ideal for many investigations of adipocyte differentiation.

Since obesity is an excessive accumulation of adipocytes, the study of adipogenesis and adipocyte biology is important for understanding not only the causes of the disease, but also to develop therapies for obesity. Importantly, a better understanding of adipocyte physiology is necessary for treatment of the diseases associated with obesity.

1.2 Adipocyte Proteins

PPAR γ

PPAR γ is a member of the nuclear hormone receptor family, a group of transcription factors which are activated by small, lipophilic ligands (Egea *et al.*, 2000). PPAR γ was discovered as a transcription factor that bound to the enhancer of aP2 (Tontonoz *et al.*, 1994a) and is known to exist as four isoforms, PPAR γ 1, 2, 3, and 4, produced by differential promoter usage and alternative splicing (Zhu *et al.*, 1995; Fajas *et al.*, 1998; Sundvold *et al.*, 2001). PPAR γ 1, 2 and 3 are all expressed in adipose tissue, but PPAR γ 1 is present at low levels in multiple tissues, PPAR γ 3 is also expressed in large intestine, and PPAR γ 4 tissue distribution is not known (Tontonoz *et al.*, 1994a; Chawla *et al.*, 1994; Fajas *et al.*, 1998; Sundvold and Lien, 2001).

PPAR γ has been implicated in the regulation of systemic insulin sensitivity, particularly through the insulin-sensitizing effects of the synthetic PPAR γ ligands, thiazolidinediones (TZDs) (Lehmann *et al.*, 1995). TZDs decrease the production of TNF α and resistin, two secreted

products of adipocytes which promote insulin resistance (Peraldi *et al.*, 1997; Steppan *et al.*, 2001), promote synthesis of adiponectin, an insulin sensitizer (Yamauchi *et al.*, 2001), and improve the ability of adipose tissue to store lipid (Evans *et al.*, 2004). Direct evidence for the association between PPAR γ and insulin sensitivity comes from genetic studies showing that mutations in the ligand-binding domain of PPAR γ are associated with severe insulin resistance in humans (Barroso *et al.*, 1999).

PPAR γ is also considered a master regulator of adipocyte differentiation as PPAR γ is highly induced during adipogenesis and the ectopic expression of PPAR γ effectively converts nonprecursor fibroblast cells into adipocytes (Tontonoz *et al.*, 1994b). Although transgenic mice that only express one copy of the PPAR γ gene are more sensitive to insulin and are resistant to diet induced obesity (Miles *et al.*, 2000), the tissue-specific knockout of PPAR γ in mature fat tissue results in loss of adipose tissue mass, hyperlipidemia, and hepatic insulin resistance (He *et al.*, 2003). These findings reveal the importance of precise regulation of PPAR γ expression for whole body glucose metabolism and the development and maintenance of adipose tissue.

Regulation of PPAR γ expression occurs at the levels of transcription and protein stability. E boxes in the promoters of PPAR γ 1 and PPAR γ 3 have been characterized as sites for regulation by SREBP1 (Fajas *et al.*, 1999). Liver X receptor (LXR) upregulated PPAR γ expression during adipogenesis through an LXR site in the PPAR γ 2 promoter (Seo *et al.*, 2004). Krüppel-like factor 2 inhibits PPAR γ 2 expression in preadipocytes through two tandem Krüppel binding sites in the promoter (Banerjee *et al.*, 2002). A STAT5B binding site in the human PPAR γ 3 promoter was identified as a polymorphism governing height and lipid metabolism (Meirhaeghe *et al.*, 2003). Moreover, binding sites for C/EBP proteins have been characterized (Elberg *et al.*, 2000; Tang *et al.*, 2003). Degradation of PPAR γ protein occurs under basal conditions and following

stimulation by TZDs (Hauser *et al.*, 2000) and IFN γ (Waite *et al.*, 2001). IFN γ treatment of 3T3-L1 adipocytes also decreased PPAR γ expression by regulating synthesis of PPAR γ mRNA (Waite *et al.*, 2001). PPAR γ expression was transiently decreased following treatment of 3T3-L1 adipocytes with the gp130 cytokine, cardiotrophin-1 (CT-1) independent of the MAPK pathway (Zvonic *et al.*, 2004). Thus, signaling pathways activated by these cytokines likely contribute to the regulation of PPAR γ expression in adipocytes.

C/EBP δ

C/EBP δ is a basic-leucine zipper transcription factor and is one of six members of the C/EBP family of transcription factors (Tanaka *et al.*, 1997). Members of the C/EBP family contain the basic leucine zipper dimerization domain, as well as basic and acidic DNA binding domains (Belmonte *et al.*, 2001). C/EBP proteins form homo- and heterodimers, and all bind the same *cis*-regulatory elements in promoters (Tanaka *et al.*, 1997). C/EBP δ is expressed at a low level in most tissues (Huang *et al.*, 2004), but is rapidly induced by several cytokines and neurotransmitters (Takiguchi, 1998; Ramji and Foka, 2002). C/EBP δ , along with C/EBP β , is part of a transcriptional cascade that directs adipogenesis (Lane *et al.*, 1999). Double knockout of C/EBP β/δ inhibits fat tissue development *in vivo* and adipogenesis in cultured mouse embryonic fibroblasts (MEFs) (Tanaka *et al.*, 1997). Adipogenesis is recovered with the ectopic expression of PPAR γ in these double knockout MEFs, yet these adipocytes exhibit decreased expression of IRS-2 and GLUT4 (Yamamoto *et al.*, 2002). Thus, C/EBPs are regulators of glucose uptake and insulin sensitivity and have nonoverlapping roles in adipocytes.

Lipoprotein Lipase (LPL)

LPL is a member of the lipase family which also includes hepatic lipase and pancreatic lipase. These enzymes likely evolved from a common ancestral gene that encoded a digestive

lipase in the intestine (Kirchgessner *et al.*, 1989). LPL catalyzes the hydrolysis of serum triglycerides from very low density lipoproteins and chylomicrons for uptake of free fatty acids (Braun and Severson, 1992). LPL is present in many tissues, but the majority of the enzyme originates as a secreted protein from skeletal muscle and adipose tissue (Knutson, 2000). The functionally active form of LPL is a glycoprotein anchored by heparan sulfate proteoglycan to the surface of capillary endothelial cells and provides free fatty acids to the underlying tissues (Braun and Severson, 1992). In WAT, free fatty acids are re-esterified for storage as triglycerides, whereas in BAT the activity of LPL regulates thermogenesis (Braun and Severson, 1992). In other tissues, such as skeletal muscle and cardiac muscle, the released free fatty acids are used as a primary energy source (Braun and Severson, 1992).

LPL deficiency in humans is a rare autosomal recessive disorder resulting in fasting hypertriglyceridemia, accumulation of chylomicrons, elevated serum cholesterol and failure to thrive, but it does not cause any apparent defects in the development of adipose tissue (Brun *et al.*, 1989). The transgenic knockout of LPL in mouse results in a very similar phenotype, but the mice die within 30 hours of birth (Coleman *et al.*, 1995; Weinstock *et al.*, 1995). Moderate overexpression of LPL in skeletal muscle of obese rodents protects against diet-induced obesity (Jensen *et al.*, 1997). Yet, the white adipose tissue-specific knockout of LPL also protected obese rodents against diet-induced obesity (Weinstock *et al.*, 1997). However, high levels of LPL expression have been correlated with obesity in humans and rodents (Maggio and Greenwood, 1982; Greenwood, 1985). Thus, the tissue-specific expression of LPL has profound effects on energy partitioning.

Regulation of LPL is complex and occurs at the levels of transcription, translation, and post-translational processing, indicative of the importance of LPL in meeting the varying

metabolic demands in tissues (Braun and Severson, 1992). LPL is sensitive to regulation by steroids, temperature, feeding, and catecholamines in a tissue-specific manner (Greenwood, 1985; Braun and Severson, 1992). Fasting decreases activity of LPL in WAT, but increases LPL activity in heart (Braun and Severson, 1992). Refeeding and insulin have been shown to increase LPL activity in WAT (Braun and Severson, 1992). Furthermore, cold exposure activates LPL in BAT (Braun and Severson, 1992). Taken together, these findings indicate that the tissue-specific regulation of LPL expression is critical for lipid metabolism and homeostasis. In addition, LPL activity and expression are regulated by cytokines, $\text{TNF}\alpha$, $\text{IFN}\alpha$, and $\text{IFN}\gamma$, in 3T3-F442A adipocytes (Doerrler *et al.*, 1994). In spite of these findings, little is known about the mechanisms of transcriptional regulation of LPL, and it is an important area of research.

Fatty Acid Synthase (FAS)

When energy intake exceeds energy expenditure, white adipose tissue stores excess calories as triglycerides. Although the majority of stored lipids come from diet, the production of long chain fatty acids from carbohydrate sources by *de novo* lipogenesis is an important contribution to total body lipid stores, particularly under the condition of a high carbohydrate diet (Sul and Wang, 1998). FAS is the central enzyme of *de novo* lipogenesis, synthesizing palmitate from malonyl CoA generated from glycolysis (Sul and Wang, 1998). Transgenic knockout of FAS in mice results in preimplantation embryonic death (Chirala *et al.*, 2003). Heterozygotes die throughout embryonic development, yet some survive through maturity (Chirala *et al.*, 2003). Thus *de novo* lipogenesis is important in embryonic development. C75, α -methylene- γ -butyrolactone, is a pharmacological allosteric inhibitor of FAS (Kuhajda *et al.*, 2000). C75 administration results in weight loss in rodents, but this effect is more likely mediated by decreased food intake due to increased production of an anorexin, neuropeptide Y,

in the hypothalamus, than the direct inhibition of FAS in adipose tissue (Loftus *et al.*, 2000). In cultured 3T3-L1 cells, C75 decreases the activity of GPDH, accumulation of lipid and expression of PPAR γ , hallmarks of adipogenesis, strongly suggesting that FAS activity is an important determinant of adipogenesis (Liu *et al.*, 2004). These findings underscore the importance of FAS activity and *de novo* lipogenesis in adipocyte function.

FAS is a large multifunctional monomer composed of seven catalytic domains: β ketoacyl synthetase, acetyl-CoA transacylase, malonyl-CoA transacylase, dehydratase, enoyl reductase, ketoacyl reductase and thioesterase, in addition to an acyl carrier domain (Smith, 1994). The animal FAS gene is large, and the rat FAS gene is comprised of 43 exons and 42 introns (Sul and Wang, 1998). The FAS monomer likely resulted from gene fusion, as the equivalent enzyme in bacteria and plants is a multi-enzyme complex (Smith, 1994). Fusion of the separate genes likely evolved as spatial proximity of catalytic domains, stabilization of the macromolecular structure, and perhaps most importantly, the coordinate regulation of the partial catalytic activities were optimized (Smith, 1994).

The regulation of FAS occurs primarily at the level of transcription, as no known *in vivo* allosteric inhibitors have been identified (Sul and Wang, 1998). FAS is exquisitely sensitive to nutritional and hormonal regulation (Sul and Wang, 1998). Expression increases following feeding and in response to increased insulin levels (Moustaid *et al.*, 1993; Soncini *et al.*, 1995; Kim *et al.*, 1998; Rufo *et al.*, 1999; Moon *et al.*, 2000). Multiple insulin response elements have been identified and include E boxes, bound by upstream stimulatory factor-1 (USF-1) (Wang and Sul, 1995) and steroid response elements (SRE) bound by SREBP1 (Kim *et al.*, 1998). Thyroid hormone, which increases during refeeding, increases FAS activity and expression in cultured 3T3-L1 adipocytes and rat adipose tissue (Moustaid and Sul, 1991; Blennemann *et al.*, 1995).

Moreover, a thyroid hormone response element which activates transcription of FAS has been characterized in the human FAS promoter (Xiong *et al.*, 1998). The repression of FAS expression in cultured rat white adipose tissue has also been demonstrated with fasting and in response to glucagon, a hormone which raises intracellular levels of cAMP (Foufelle *et al.*, 1994). Although an inverted CCAAT box, a cAMP response element, has been identified, it is not clear how cAMP regulates FAS expression, as the inverted CCAAT box is bound by basal transcription factors which are not regulated by cAMP or glucagon (Rangan *et al.*, 1996; Roder *et al.*, 1997). In two independent studies, dexamethasone was shown to increase the expression of FAS in cultured rat adipocytes (Reul *et al.*, 1997) and the activity of the FAS promoter in adipose tissue (Freak and Moon, 2003). Yet, inhibition of FAS expression by dexamethasone has also been observed (Foufelle *et al.*, 1994). The discrepancy in these effects may depend on the nutritional state of the organism. GH, an important developmental cytokine, abrogates the induction of FAS expression by insulin and down regulates basal expression of FAS in 3T3-F442A cells (Yin *et al.*, 2001a; 2001b), yet a GH responsive region of the FAS promoter has not been identified. The identification of these and other regulatory elements in the FAS promoter are indicative of the complex and precise regulation of FAS and *de novo* lipogenesis in adipose tissue.

1.3 JAK/STAT Signaling

Some extracellular signaling molecules transmit signals through transmembrane receptors to the Janus kinase (JAK)/signal transducers and activators of transcription pathway (STAT) to regulate gene expression. The JAK/STAT pathway integrates multiple intercellular signals to initiate cell and tissue responses to stress, modulate development and regulate homeostasis.

The JAK/STAT pathway is the predominant signaling cascade for cytokines and is initiated by the binding of a ligand to its cognate receptor (reviewed in Kisseleva *et al.*, 2002). Cytokine receptors lack intrinsic kinase activity, but undergo conformational changes in the cytoplasmic domains, which activate the associated JAK. JAKs are non-receptor tyrosine kinases which transphosphorylate the tyrosine residues on the cytoplasmic portion of the receptor that serve as STAT-docking sites. STAT proteins are latent transcription factors, residing in the cytoplasm and bind to the phosphorylated tyrosines through their src homology 2 (SH2) domain, bringing the STATs within close proximity to the JAKs. The JAKs then phosphorylate the STATs on a single, conserved tyrosine located just carboxy terminal to the SH2 domain. Upon phosphorylation, STATs dissociate from the receptor and form either homodimers or heterodimers with other phosphorylated STAT proteins. STAT dimers rapidly translocate to the nucleus to bind specific DNA elements to modulate transcription.

JAKs are a family of kinases named after Janus, the two-faced Roman god of doors and gates, for their two hallmark domains, the catalytic domain and a noncatalytic pseudokinase domain (reviewed in Leonard and O'Shea, 1998). Four members have been identified, JAKs 1-3 and Tyk2, and are expressed ubiquitously, except for JAK3, which has limited expression in natural killer cells, thymocytes, B cells, myeloid cells, vascular smooth muscle cells and endothelium. The JAK homology (JH) 1 domain is the catalytic domain with all the features and subdomains of other tyrosine kinase domains. JH2 is the pseudokinase domain which has very similar features of a tyrosine kinase domain and may regulate JAK catalytic activity. The domains JH3-7 are relatively divergent among JAK proteins and have been implicated in receptor association.

Seven STATs have been identified, STATs 1, 2, 3, 4, 5A, 5B, and 6 which range in size from 750 to 850 amino acids in length (Kisseleva *et al.*, 2002). The STAT family likely resulted from duplication of a primordial gene, as indicated by the presence of STAT homologues in lower eukaryotes: *Dictyostelium*, *C. elegans* and *Drosophila* (Miyoshi *et al.*, 2001), and by the clustering of *stat* genes on chromosomes (Choi *et al.*, 1996; Lin *et al.*, 1996; Leek *et al.*, 1997; Yamamoto *et al.*, 1997).

STATs are comprised of 5 conserved domains and a variable C-terminal domain (Chen *et al.*, 1998). The amino terminus is comprised of 130 amino acids, which serve to promote the cooperative binding to multiple consensus sites within a promoter (Vinkemeier *et al.*, 1996; 1998). The coiled-coil domain of 4 α -helices forms a hydrophilic surface for interaction with other proteins (Chen *et al.*, 1998). The DNA binding domain (DBD) is a β -barrel and is similar to the DBDs of NF- κ B and p53 (Chen *et al.*, 1998). This domain includes a nuclear export signal (Mowen *et al.*, 2000; McBride *et al.*, 2000) in addition to a non-canonical nuclear localization sequence (Melen *et al.*, 2001). The linker domain contacts the DBD and the SH2 domain and may facilitate conformational changes of the STAT protein (Chen *et al.*, 1998). The SH2 domain is comprised of two anti-parallel β -strands contacting two α -helices and binds to a phosphorylated tyrosine within a specific motif, mediating contacts with other proteins (Chen *et al.*, 1998). The conserved tyrosine is a few amino acids distal to the SH2 domain. Phosphorylation of this tyrosine is necessary for activation, dimerization and nuclear translocation (Ihle, 2001). The domain that is divergent for each STAT is the transcriptional activation domain (TAD). This domain is thought to make contact with other transcriptional regulators that modify the chromatin or contact the transcriptional machinery (Kisseleva *et al.*, 2002).

STAT1 was the first discovered STAT, identified as the transcription factor that bound IFN α/β recognition sequences upon activation by IFN α/β (Levy *et al.*, 1988). STAT1 can function as a homodimer, a heterodimer with STAT3 or as a trimer with STAT2 and p48/interferon regulated factor 9 (IRF-9) (Kisseleva *et al.*, 2002). The transgenic knockout of STAT1 in mouse indicated that this protein has a crucial role in the innate immune response (Meraz *et al.*, 1996). STAT1^{-/-} mice exhibited defective IFN-dependent immune response to viral and microbial infection and were defective in chondrocyte proliferation (Meraz *et al.*, 1996).

STAT3 was initially identified as acute phase response factor (APRF; Akira *et al.*, 1994) which bound to IL-6 response elements/STAT induced elements (IRE/SIE; Lutticken *et al.*, 1994). STAT3 expression is nearly ubiquitous. Transgenic knockout of STAT3 in mice resulted in lethality early in embryogenesis, around embryonic day 7.5 (Takeda *et al.*, 1997) indicating an early role for STAT3 in development. Tissue-specific knockouts have indicated roles for STAT3 in T cells (Takeda *et al.*, 1998), macrophages (Takeda *et al.*, 1999), mammary gland (Chapman *et al.*, 1999), liver (Alonzi *et al.*, 2001), and skin (Sano *et al.*, 1999).

STAT5 proteins were first identified as mammary gland factor (MGF), a protein from mouse mammary gland that bound to the β -casein promoter (Schmitt-Ney *et al.*, 1991). It was subsequently determined that MGF was two closely related proteins, STAT5A and STAT5B (Wakao *et al.*, 1994), which are expressed in all tissues (Kisseleva *et al.*, 2002). Transgenic deletion of STAT5 resulted in defective prolactin-dependent mammary gland development but also significantly reduced fat pad size (Teglund *et al.*, 1998). Thus, although a major role for STAT5 proteins is in the regulation of mammary tissue development, STAT5 proteins likely modulate adipocyte function. During differentiation of 3T3-L1 adipocytes, expression levels of

STAT5A and 5B were highly induced (Stewart *et al.*, 1999). Moreover, ectopic expression of STAT5A has been shown to confer adipogenesis in two different nonprecursor cell lines (Floyd and Stephens, 2003).

STAT6 is ubiquitously expressed and has a role in acquired immunity. The STAT6 knockout mouse exhibited lower numbers of Th2 cells and a failure of IL-4 to induce expression of major histocompatibility factor II, IL-4 receptor or immunoglobulin E receptor (Kaplan *et al.*, 1996).

STAT action may be regulated at the level of the receptor, JAK or STAT. Modification of STATs is a mechanism of regulation; in addition to the phosphorylation of tyrosine, STATs may be dephosphorylated, serine phosphorylated, ubiquitylated, acetylated, methylated, or SUMOylated (Kisseleva *et al.*, 2002). Furthermore, STATs 1, 3, 5A, 5B, and 6 are naturally expressed as splice variants or are proteolytically cleaved to produce truncations that likely function as dominant negative regulators of STATs (Kisseleva *et al.*, 2002).

SHP1 and 2 are phosphatases that negatively regulate JAK activity and are constitutively expressed proteins that have been shown to negatively regulate cytokine signaling (Kisseleva *et al.*, 2002). These phosphatases have SH2 domains which are likely involved in recruitment to the phosphorylated receptor or JAK. Another phosphatase identified as a regulator of JAK/STAT signaling is CD45, a membrane spanning protein with a well-characterized role in T cell signaling (Alexander *et al.*, 2000). In addition, protein tyrosine phosphatase- ϵ C, is induced by cytokines and dephosphorylates a JAK to specifically downregulate STAT3 signaling (Tanuma *et al.*, 2000).

Members of the suppressors of cytokine signaling (SOCS) family are induced by STATs and directly interact with STATs to attenuate signaling in a negative feedback loop (reviewed in

Krebs and Hilton, 2001). SOCS proteins are rapidly induced upon cytokine stimulation and STAT activation. There are eight members of the family, cytokine-inducible SH2 protein (CIS) and SOCS 1-7, which contain two consensus elements, an SH2 domain and a C-terminal SOCS box, in addition to an N-terminal variable region. The SOCS box is a common motif among more than 40 proteins, the majority of which have unknown functions, although it has been hypothesized that the SOCS box could function as an adapter for E3 ubiquitin ligases (Kile *et al.*, 2002). SOCS proteins may regulate cytokine signaling by multiple mechanisms. SOCS-1 has been shown to interact with JAKs via the activation loop blocking ATP binding, and thus, inhibiting catalytic activity (Nicholson *et al.*, 1999). SOCS-3 has been found to bind receptors without inhibiting JAK kinase activity (Nicholson *et al.*, 1999). CIS also binds to receptors and prevents recruitment of STATs (Yoshimura *et al.*, 1995).

Another family of proteins that negatively regulate STATs is the protein inhibitors of activated STAT (PIAS) proteins, which are constitutively expressed. Five PIAS proteins have been identified: PIAS1, an inhibitor of STAT1 (Liao *et al.*, 2000); PIAS3, an inhibitor of STAT3 (Chung *et al.*, 1997); PIASx α and PIASx β , regulators of androgen receptor, as well as TGF β /Smad signaling (Kotaja *et al.*, 2000, Ohshima and Shimotohno, 2003), and PIASy (Liu *et al.*, 2001). PIAS 1, 3, x and y have been implicated as small ubiquitin like molecule-1 (SUMO-1) tethering proteins and likely act to bring SUMOylation targets in proximity to the SUMO conjugase, ubc9 (Kotaja *et al.*, 2002). SUMOylation of transcription factors results in activation of some, regulation of others and altered cellular localization (Eloranta *et al.*, 2002). The specific mechanisms for these effects are not yet known. STAT1 has recently been demonstrated to be a substrate for PIASx α -mediated SUMOylation (Rogers *et al.*, 2003).

STATs have important roles in responses to stress, particularly immune responses. An important area of research is identifying targets of transcriptional modulation by STATs, particularly within the context of cooperativity with other transcription factors. There is also much to be understood of the crosstalk between signaling pathways. Elucidation of STAT function will be an important facet of cytokine signaling. Characterizing STAT activity and interactions with other proteins could yield solutions to human diseases.

STATs were first implicated in energy balance with the finding that the adipocyte-synthesized hormone leptin activates STATs 3, 5 and 6 in the hypothalamus (Tartaglia *et al.*, 1995, Ghillardi *et al.*, 1996). Furthermore, the discovery that STATs 1, 3, 5A, 5B, and 6 are expressed in adipocytes has implicated STAT proteins as regulators of adipocyte function (Stephens *et al.*, 1996). STATs 1, 5A, and 5B are highly induced during differentiation; moreover, dysregulation of adipogenesis attenuates induction of these STATs (Stewart *et al.*, 1999). In the *agouti* mouse model of obesity the expression of STATs 1 and 3 are upregulated (Mynatt and Stephens, 2001). Furthermore, ectopic expression of STAT5A in nonprecursor cells confers the ability to differentiate into fat cells (Floyd and Stephens, 2003). Hence, STAT proteins may modulate multiple aspects of adipocyte biology and the expression of adipocyte proteins.

1.4 Cytokines

IFN γ

Interferon- γ (IFN γ) is a cytokine that is primarily known for its roles in immunological responses but has also been shown to affect fat metabolism and adipocyte gene expression. In adipocytes, IFN γ treatment results in decreased lipoprotein lipase activity (LPL) and increased rate of lipolysis (Doerrler *et al.*, 1994). In 3T3-F442A adipocytes, exposure to IFN γ results in

decreased expression of LPL and FAS. Also, in various rodent preadipocyte cell lines, IFN γ inhibits the differentiation of preadipocytes (Grossberg and Keay, 1980; Keay and Grossberg, 1980; Gregoire *et al.*, 1992). Acute IFN γ treatment of cultured and native rat adipocytes results in a dose- and time-dependent activation of STATs 1 and 3 (Stephens *et al.*, 1998). Moreover, studies have linked IFN γ and insulin resistance during viral infections (Koivisto *et al.*, 1989; Khanna *et al.*, 1999). IFN γ therapy of cancer patients has been associated with the development of hyperglycemia (Shiba *et al.*, 1998).

LIF

Leukemia inhibitory factor (LIF) is a member of the gp130 cytokine family, cytokines which are unrelated by sequence, but are structurally similar and share a common receptor, gp130 (Robinson *et al.*, 1994, Kishimoto *et al.*, 1995). LIF has pleiotropic actions which include maintaining totipotency of embryonic stem cells (Williams *et al.*, 1988), enhancing survival of peripheral neurons (Murphy *et al.* 1991) and oligodendrocytes (Butzkueven *et al.*, 2002), and promoting bone formation (Dazai *et al.*, 2000). LIF also has multiple effects on adipocytes and adipose tissue. Initially, LIF was characterized as an inducer of wasting weight loss in mice engrafted with a melanoma cell line that overproduces LIF (Mori *et al.*, 1989). Subsequently, LIF was found to inhibit lipoprotein lipase (LPL) expression and activity in 3T3-L1 and 3T3-F442A adipocytes but does not affect the rate of lipogenesis (Marshall *et al.*, 1994). In addition, adipogenic effects of LIF have been indicated by enhanced activity of glycerol-phosphate dehydrogenase and accumulation of lipid in Ob1771 cells during adipogenesis (Aubert *et al.*, 1999). However, other findings have indicated that LIF prevents adipogenesis in bone marrow stromal cells (Gimble *et al.*, 1994). Thus, it is likely that the effects of LIF on adipocytes vary with the developmental stage of the cells or tissue.

CT-1

Cardiotrophin-1 (CT-1) is also a member of the gp130 cytokines. Cytokines in the gp130 family exhibit functional redundancy as they signal through shared receptor components (Kishimoto *et al.* 1995). Like LIF, CT-1, ciliary neurotrophic factor (CNTF), and oncostatin M (OSM) bind the LIF receptor (LIFR), in addition to the common receptor, gp130 (Baumann *et al.* 1993, Wollert *et al.* 1996). Because these cytokines signal through common elements that are ubiquitously expressed, many of their effects are similar in various tissues. CT-1 was cloned from a cDNA library of embryoid bodies screened for clones that were capable of inducing cardiomyocyte hypertrophy (Pennica *et al.*, 1995). The clone encoded a novel 21.5 kD protein, which activated morphological and molecular markers of a hypertrophic response in culture, but also had cardioprotective effects (Pennica *et al.*, 1995; Sheng *et al.*, 1996). It promoted the growth of myocytes in length and the serial organization of sarcomeres, the same effects observed with ventricular volume overload (Wollert *et al.*, 1996). Moreover, the genes activated by CT-1 administration appeared to be a reactivation of the pattern of gene expression of the developing heart (Wollert *et al.*, 1996). CT-1 effects parallel those of LIF in the heart and other tissues. Treatment with CT-1 *in vitro* inhibits growth of mouse myeloid leukemic M1 cells, modulates sympathetic neuron transmitter phenotype, inhibits differentiation of embryonic stem cells, and induces acute phase protein expression in hepatocytes (Pennica *et al.*, 1995; 1996, Peters *et al.*, 1995). Since the expression of LIFR and gp130 are ubiquitous, the specificity of the targets may be defined by the expression of CT-1 and LIF (Ip *et al.*, 1993). LIF is expressed at low levels in adult tissues (Robertson *et al.*, 1993); however, CT-1 is highly expressed in many tissues in adults and thus, there are likely pleiotropic effects *in vitro* and *in vivo* (Pennica *et al.*, 1995; 1996; Ishikawa *et al.*, 1996).

PRL

Prolactin (PRL) is a peptide hormone primarily known for its role in mammary gland development during lactation, but it has been shown to have pleiotropic effects in a variety of tissues (Bole-Feysot *et al.*, 1998). PRL activates multiple signal transduction pathways, including MAPK (Avruch *et al.*, 1994) and phosphatidylinositol-3 kinase (Bailey *et al.*, 2004); however, the JAK/STAT pathway is the predominant signaling cascade activated by PRL. The PRL receptor (PRL-R) dimerizes upon binding by PRL, activating an associated JAK. The regulation of mammary tissue by PRL is well-characterized, but there is evidence of a role in adipose tissue. PRL-R is expressed in both mouse (Ling *et al.*, 2000) and human adipose tissue (Ling *et al.*, 2003) and is induced during adipogenesis of bone marrow stromal cells (McAveney *et al.*, 1996). In addition, ectopic expression of the PRL-R in NIH-3T3 cells resulted in efficient adipocyte conversion and activation of the aP2 promoter in a PRL-dependent manner (Nanbu-Wakao *et al.*, 2000). Taken together, these observations strongly suggest a role for PRL in the modulation of adipocyte function. Furthermore, the occurrence of obesity has been correlated with hyperprolactinomas (Greenman *et al.*, 1998). In opposition to these adipogenic effects, PRL has been shown to induce lipolysis in rabbits (Fortun-Lamothe *et al.*, 1996) and mouse adipose tissue explants (Fielder and Talamantes, 1987). In addition, studies have shown that PRL reduces LPL activity in cultured human adipocytes (Ling *et al.*, 2003), and the activity of LPL and FAS in adipose tissue of lactating mice (Flint *et al.*, 1981). Thus, PRL exerts adipogenic and anti-lipogenic effects on adipose tissue.

1.5 Rationale

Five members of the STAT transcription factor family are expressed in adipocytes; STATs 1, 5A, and 5B are highly induced during adipogenesis, and STATs 3 and 6 are expressed

in preadipocytes and adipocytes (Stephens *et al.*, 1996; Stewart *et al.*, 1999). STAT5A promotes adipogenesis in nonprecursor cells, and thus STAT5A may be a component of the transcriptional cascade governing adipogenesis (Floyd and Stephens, 2003). However, a function for STAT proteins is not known in adipocytes. The identification of transcriptional targets of STATs will provide some insight into the function of STATs in mature adipocytes. Thus, we have characterized STAT binding sites in the promoters of genes which are regulated by STAT-activating cytokines. STAT binding sites have been found in the promoters of PPAR γ 2, LPL, FAS and C/EBP δ , suggesting that the expression of these genes is modulated by STATs *in vivo*.

CHAPTER 2: EXPERIMENTAL PROCEDURES

2.1 Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and leukemia inhibitory factor (LIF) were purchased from Invitrogen. Murine interferon- γ (IFN γ) was purchased from Boehringer Mannheim. Fetal bovine serum (FBS) was purchased from Atlanta Biologicals, and calf serum was purchased from Biosource. Bovine serum, actinomycin D, cycloheximide, and GH were purchased from Sigma. Foxo1, STAT1, STAT3, and highly phospho-specific STAT5 (Tyr⁶⁹⁴) antibodies were purchased from Upstate Biotechnology; STAT3, STAT5A, PPAR γ , and SREBP1 antibodies were purchased from Santa Cruz; STAT3, highly phospho-specific STAT3 (Tyr⁷⁰⁵), STAT 5 (Y⁶⁹⁴), JAK1(p-Y) and JAK2(p-Y) antibodies, and FAS antibody were purchased from BD transduction laboratories. LPL antibody was purchased from Research Diagnostics, Inc, and highly phospho-specific STAT1 (Tyr⁷⁰¹) was purchased from Biosource. [α -³²P] dCTP and [α -³²P] dATP were both purchased from Perkin-Elmer and Amersham Biosciences. Deoxynucleotide thymine triphosphate, dATP, and dGTP were purchased from Amersham Biosciences. DNA polymerase I large (Klenow) fragment and U0126 were purchased from Promega. Oligonucleotides were purchased from Integrated DNA Technologies.

2.2 Cell Culture

Murine 3T3-L1 preadipocytes were plated and grown to 2 days post-confluence in DMEM containing 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutylmethylxanthine, 1 μ M dexamethasone, and 1.7 μ M insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% FBS, and the cells were maintained in this

medium until utilized for experimentation. The adipocyte phenotype was attained following 120 hours of differentiation.

2.3 Preparation of Whole Cell Extracts

Cell monolayers were rinsed with PBS and then harvested in a nondenaturing buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions.

2.4 Preparation of Nuclear and 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, 3 mM MgCl₂, 5 μ M dithiothreitol, 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, and 2 mM sodium vanadate. Igepal CA-630 (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 min. 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. 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, 5 μ M dithiothreitol, 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, 2 mM sodium vanadate,

and 25% glycerol. Nuclei were extracted for 30 min on ice. The samples were subjected to centrifugation at 10,000 rpm at 4°C for 10 min. Supernatants containing nuclear extracts were analyzed for protein content, using a BCA protein assay kit (Pierce).

2.5 Gel Electrophoresis

Proteins were separated in 7.5% polyacrylamide (National Diagnostics) gels containing sodium dodecyl sulfate (SDS) according to Laemmli (1970) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk overnight at 4°C. Results were visualized with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and enhanced chemiluminescence (Pierce).

2.6 RNA Analysis

Total RNA was isolated from cell monolayers with Trizol (Invitrogen) according to manufacturer's instructions with minor modifications. For Northern blot analysis, 15 µg of total RNA was denatured in formamide and electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Zeta Probe-GT (Bio-Rad), cross-linked, hybridized, and washed as previously described (Stephens and Pekala, 1992). The blots for FAS were transferred in a buffer containing 75 mM sodium citrate tribasic, 10 mM NaOH and 750 mM NaCl. Probes were labeled by random priming using Klenow fragment and [α -³²P] dCTP or using Strip-EZ DNA (Ambion) and [α -³²P] dATP according to manufacturer's instructions to generate probes that were easily removed from the membrane.

2.7 Electrophoretic Mobility Shift Assay

Double-stranded oligonucleotides were 3'-end labeled with 20 µCi [32 P] (400-800 Ci/mmol) dCTP and Klenow fragment. End-labeled oligonucleotides were purified using a

Microspin G-25 column, according to manufacturer's instructions (Amersham Biosciences). Specific activity of the oligonucleotides was determined by scintillation counting. Nuclear extracts were incubated with the end-labeled oligonucleotides (50,000 cpm/ μ L) for 30 min on ice in a binding buffer containing 15 μ M EDTA, 40 mM KCl, 3.75 mM Hepes, 5 mM MgCl₂, 1.2% Ficoll, and 3 μ g poly dIdC (Amersham Biosciences), as previously described by Ritzenthaler and colleagues (1991). The samples were loaded on a pre-run (1 h, 100 V at 4°C) 6% acrylamide/bisacrylamide TBE gel containing 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. For supershift analysis, nuclear extracts were preincubated with 4 μ g of antibody for 1 h at room temperature. For cold competition, nuclear extracts were incubated with unlabeled oligonucleotide for 15 min on ice prior to incubation with the labeled probe. The gels were run at 20 mA for approximately 2 h. The gels were dried at 80°C for 1 h under a vacuum and then exposed to Kodak BioMax MS film with a Kodak BioMax high energy intensifying screen.

2.8 Constructs

The PPAR γ 2 promoter (-609 to +52)/luciferase reporter construct and C/EBP α expression construct were generous gifts from Dr. Jeffrey Gimble. The rat FAS promoter (-250 to +65)/luciferase construct was generously provided by Dr. Steven Clarke. The rat FAS promoter (-1594 to +65)/luciferase and (-700 to +65)/luciferase constructs were generously provided by Dr. Peter Tontonoz. The PPAR γ 2 promoter (-609 to +65)/luciferase construct and the FAS (-1594 to +65)/luciferase construct were mutated at positions -217 and -212, and -901 and -902, respectively, within the STAT binding sites using the QuikChange Site Directed Mutagenesis Kit, according to manufacturer's instructions (Stratagene). The following oligonucleotide and corresponding anti-sense oligonucleotide were used to alter the STAT binding site with the altered bases underlined: PPAR γ /GAC AAT GTA GCA ACG TTC TCC TCG TAA TGT ACC

AAG TC and FAS/GGG AGG GTG AGG GTC AAG GAA ACC AGC AAC TCA GG.

Sequence analysis was performed to confirm the presence of the mutated bases using Big Dye Terminator Extension Reaction (ABI Prism). The minimum promoter thymidine kinase (TK) renilla vector was purchased from Promega.

2.9 Transient Transfections

3T3-L1 preadipocytes were transiently cotransfected with the various promoter constructs and the TK/renilla vector or SV40/ β -galactosidase construct to control for transfection efficiency, as previously described (Hogan and Stephens, 2001; Zvonic *et al.*, 2004), using FuGENE 6 (Roche Molecular Biochemicals) or Polyfect (Qiagen) according to manufacturer's instructions. Cell lysates were analyzed for firefly and renilla luciferase activity or β -galactosidase activity using the Dual Luciferase Reporter Assay System or the Luciferase Assay System and the β -galactosidase Enzyme System (Promega). Relative light units (RLU) were determined by dividing firefly luciferase activity by renilla luciferase activity or β -galactosidase activity. Results are given as +/- standard deviation.

2.9 Determination of [3 H]2-Deoxyglucose Uptake

The assay of [3 H]2-deoxyglucose was performed as previously described (Stephens and Pekala, 1991). Briefly, mature 3T3-L1 adipocytes were serum deprived for 4 h and then incubated in the presence or absence of insulin (44 nM). Glucose uptake was initiated by addition of [3 H]2-deoxyglucose at a concentration of 0.1 mM 2-deoxyglucose in 1 μ Ci of [3 H]2-deoxyglucose in Krebs-Ringer-Hepes buffer. Glucose uptake is reported as [3 H] radioactivity, corrected for nonspecific diffusion and normalized to total protein content as determined by BCA analysis. Uptake measurements were performed in triplicate under conditions where hexose uptake was linear.

CHAPTER 3: IDENTIFICATION AND CHARACTERIZATION OF A STAT1 BINDING SITE IN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ 2 PROMOTER*§

3.1 Introduction

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a member of the nuclear hormone receptor family and exists as four isoforms, three of which are known to be highly expressed in adipocytes (Zhu *et al.*, 1995; Fajas *et al.*, 1998; Sundvold *et al.*, 2001). PPAR γ is a transcription factor which governs adipogenesis, modulates systemic insulin sensitivity, and contributes to energy metabolism (Tontonoz *et al.*, 1994b; Lehmann *et al.*, 1995; He *et al.*, 2003). Interferon- γ (IFN γ) decreases PPAR γ expression by targeting PPAR γ to the proteasome for degradation and blocking synthesis of PPAR γ in a manner that is independent of new protein synthesis (Waite *et al.*, 2001). IFN γ is a cytokine with anti-adipogenic effects and stimulates lipolysis in mature cultured adipocytes (Keay and Grossberg, 1980; Grossberg and Keay, 1980; Gregoire *et al.*, 1992; Doerrler *et al.*, 1994). Since IFN γ is a potent activator of signal transducers and activators of transcription (STATs) 1 and 3 in adipocytes (Stephens *et al.*, 1998), we have hypothesized that IFN γ -activated STATs negatively regulate the transcription of PPAR γ .

In this investigation, we have identified a site in the PPAR γ 2 promoter that is responsive to IFN γ treatment and is bound by STAT1. In a recent study, it was demonstrated that cardiotrophin-1 (CT-1), a gp130 cytokine, transiently downregulated expression of PPAR γ in

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§ Reprinted from Journal of Biological Chemistry, 279, Zvonic *et al.*, Effects of cardiotrophin-1 (CT-1) on adipocytes, 47572-47579, 2004, with permission from ASBMB.

3T3-L1 adipocytes (Zvonic *et al.*, 2004). Therefore, we have also investigated the ability of two gp130 cytokines, leukemia inhibitory factor (LIF) and CT-1, to stimulate binding by STAT proteins to this site in the PPAR γ 2 promoter. Although both LIF and CT-1 stimulate binding by STAT1 to the site in the PPAR γ 2 promoter, LIF did not affect PPAR γ mRNA levels. These studies demonstrate that the PPAR γ 2 promoter contains a highly specific STAT1 binding site and we propose that the repression of PPAR γ transcription induced by IFN γ and CT-1 is mediated by STAT1 homodimers.

3.2 Results

Our recent studies demonstrated that acute IFN γ treatment of adipocytes resulted in a repression of PPAR γ transcription that was independent of new proteins synthesis (Waite *et al.*, 2001). Therefore, we examined the PPAR γ promoter sequences currently available in GenBank for IFN γ responsive elements. We focused our search on IFN γ -activated sequence (GAS)/STAT induced element (SIE)-like elements (TTCNNNGAA) that are known to mediate IFN γ -sensitive regulation in a STAT-dependent manner (Shuai *et al.*, 1992; Khan *et al.*, 1993). As shown in Table 3.1, our analysis revealed three putative elements in the PPAR γ 2 promoter that resembled GAS elements.

Table 3.1 Potential STAT binding sites in the PPAR γ 2 promoter

Gene	Position	Sequence	IFN γ Responsive
Consensus GAS/SIE		TTC NNN GAA	
PPAR γ 2	-107 to -95	T TTC TGT GTT TAT	No
PPAR γ 2	-423 to -412	T TTG GCC AAA TA	No
PPAR γ 2	-221 to -207	C GTT TTC CTT GTA AT	Yes
PPAR γ 2	mutant -221	C GTT CTC CTC GTA AT	No

Electrophoretic mobility shift assays (EMSAs) were performed with these labeled sites and extracts isolated from 3T3-L1 adipocytes that were untreated or exposed to an IFN γ

treatment for 15 min. The responsiveness of these sites to IFN γ was examined by EMSA by using cytosolic and nuclear extracts. As shown in Fig 3.1, the -221 to -207 site of the PPAR γ 2 promoter was bound by two protein complexes which we classified as Shift #1 and Shift #2. Notably, this site was only shifted by nuclear proteins and not by cytosolic proteins. Moreover, Shift #1 was highly responsive to IFN γ treatment. The free unbound probe is not shown in the figure. The other GAS-like elements we identified in the PPAR γ 2 promoter at -423 to -412 and -107 to -95 were not shown to be responsive to IFN γ treatment (Fig 3.1).

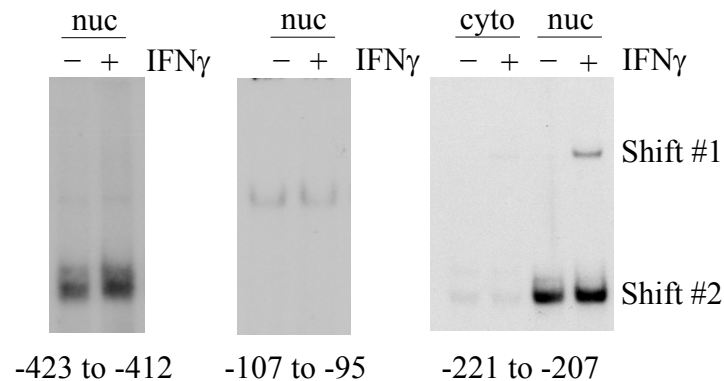


Figure 3.1 The identification of an IFN γ -sensitive element in the PPAR γ 2 promoter.

Cytosolic and nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) for 15 min with IFN γ . The extracts were incubated with the 32 P-labeled oligonucleotide of the -221 to -207 site of the PPAR γ 2 promoter for analysis by EMSA. This is a representative experiment independently performed three times.

The specificity of binding to this site was demonstrated by the use of cold competitor and mutant oligonucleotides. The binding of nuclear extracts to radiolabeled -221 to -207 site was successively competed with increasing concentrations (400 pg/ μ l to 1 μ g/ μ l) of cold competitor (Fig 3.2A and Fig 3.2B). The specificity of both Shift #1 and Shift #2 is indicated with the use of cold competitor. However, a sequence which also resembled the consensus GAS element, -107 to -95 of the PPAR γ 2 promoter, did not compete the binding by the protein complex to the

labeled -221 to -207 site (Fig. 3.2B). Also, a mutant -221 to -207 sequence (Table 1) did not compete for binding to Shift #1, but it did reduce some of the binding to Shift #2.

To assess whether STAT proteins were components of the protein complexes that bind to the PPAR γ 2 promoter following IFN γ treatment, we performed supershift analysis. Prior to EMSA analysis, nuclear extracts were incubated with antibodies directed against STATs 1, 3, or 5A. We investigated the binding of these three STATs because they are the only STATs present in the nucleus under these conditions. As shown in Fig 3.2A, the STAT1 antibody completely

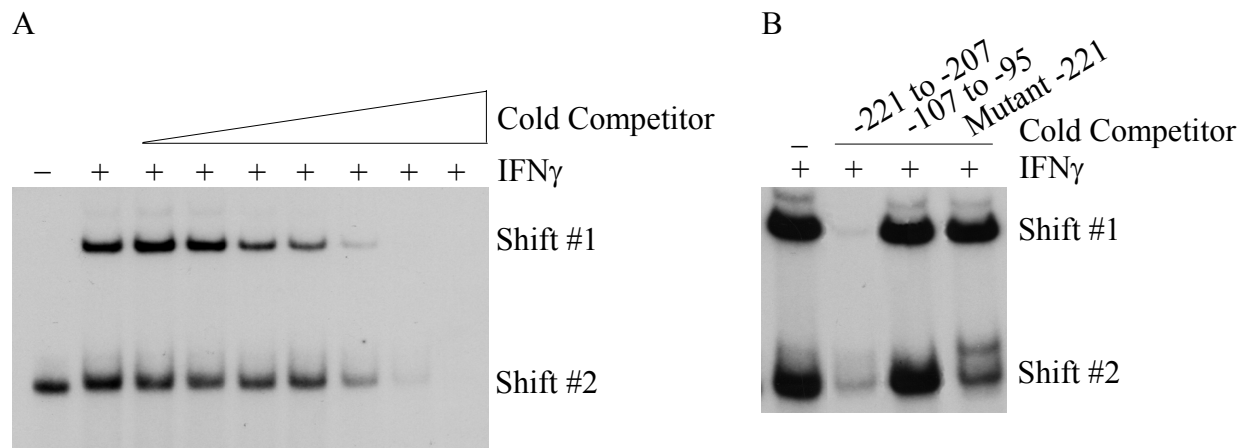


Figure 3.2 Binding to the -221 to -207 site of the PPAR γ 2 promoter is specific. (A) Nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) with IFN γ for 15 min. The extracts were preincubated with varying concentrations of unlabeled -221 to -207 oligonucleotide (3.6 nM to 8.9 μ M). The nuclear extracts were then incubated with the labeled probe. (B) Nuclear extracts from IFN γ -treated 3T3-L1 adipocytes were preincubated with unlabeled oligonucleotides (8.9 μ M) of the -221 to -207 or the -107 to -95 sites of the PPAR γ 2 promoter or with the mutant -221 to -207 site. The nuclear extracts were then incubated with the labeled -221 to -207 probe and subjected to EMSA. This is a representative experiment independently performed two times.

supershifted Shift #1, but had no effect on Shift #2. Moreover, antibodies to either STAT 3 or 5A did not supershift Shift #1. As shown in Fig 3.3B, a time course analysis revealed that binding to Shift #2 was not responsive to IFN γ (as also indicated in Figs 3.1 and 3.2), but the binding to Shift #1 was regulated by IFN γ in a time-dependent manner. Following a 15-minute

or two-hour stimulation with IFN γ , STAT1 binds to the -221 to -207 sequence. Yet after a four-hour stimulation with IFN γ , the binding by STAT1 is greatly reduced. Also, the binding to Shift #1 correlates with the presence of STAT1 in the nucleus (data not shown).

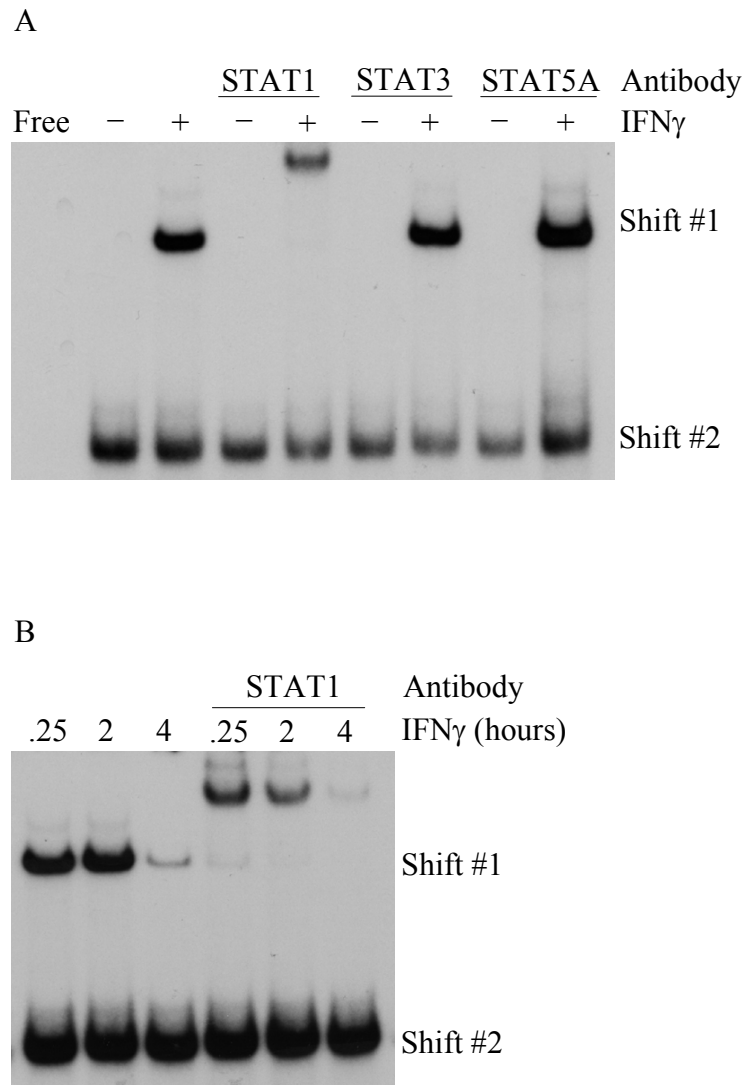


Figure 3.3 STAT1 binds to the PPAR γ 2 promoter *in vitro*. (A) Nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) with IFN γ for 15 min. For supershift analysis, the nuclear extracts were preincubated with antibodies (4 μ g) for STAT1, 3 or 5A prior to incubation with the 32 P-labeled -221 to -207 oligonucleotide. (B) Nuclear extracts were prepared from 3T3-L1 adipocytes that were treated with IFN γ for the various times indicated. The extracts were incubated with the -221 to -207 radiolabeled probe for EMSA analysis. Supershift analysis was performed by preincubating the nuclear extracts with a STAT1 antibody (4 μ g). This is a representative experiment independently performed two times.

To determine if this region of the PPAR γ 2 promoter containing the STAT1 site (-221 to -207) was sensitive to IFN γ regulation, we obtained a luciferase reporter construct with -609 to +52 base pairs of the PPAR γ 2 promoter. This construct was transiently transfected into both NIH 3T3 cells and 3T3-L1 cells. In each experiment, cells were cotransfected with an SV40/ β -galactosidase vector to control for transfection efficiency. However, the NIH 3T3 cells were also cotransfected with a C/EBP α expression in order to activate the PPAR γ 2 promoter through the C/EBP recognition site at -340 to -327 (Clarke *et al.*, 1997). As previously reported (Clarke *et al.*, 1997), cotransfection with the C/EBP α expression construct resulted in a substantial increase in luciferase activity compared to transfection of the PPAR γ 2 promoter (-609 to +52)/luciferase reporter construct alone (data not shown). As shown in Fig 3.4A, treatment of the cotransfected NIH 3T3 cells with IFN γ for two or 24 hours resulted in a discernable reduction in luciferase activity compared to untreated cotransfected NIH 3T3 cells when normalized to β -galactosidase activity. We also performed transient transfections with the PPAR γ 2 reporter construct in 3T3-L1 cells. The 3T3-L1 cells were transfected with the PPAR γ 2 promoter (-609 to +52)/luciferase reporter construct 24 hours after removal of the differentiation cocktail. As shown in Fig 3.4B, treatment with IFN γ for two hours resulted in substantial reduction in luciferase activity compared with the untreated transfected 3T3-L1 cells (Fig 3.4B). After 24 hours, basal levels of the reporter expression did not change, but there was still a detectable decrease in PPAR γ promoter activity in the IFN γ -treated cells. Transfection of the 3T3-L1 cells at different times after the induction of differentiation produced similar responses to IFN γ (data not shown). To determine the functional significance of the -221 to -207 site of the PPAR γ 2 promoter, we transfected 3T3-L1 cells with the wild type PPAR γ 2 promoter (-609 to +52)/luciferase construct

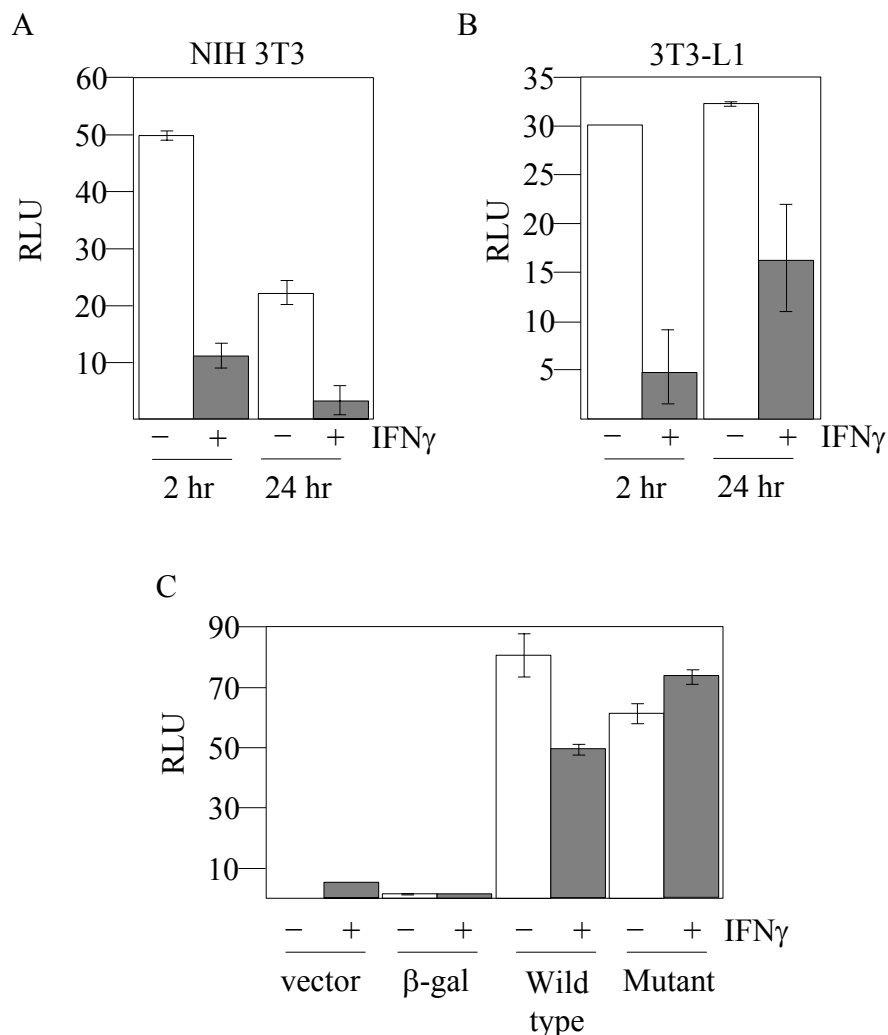


Figure 3.4 IFN γ modulates the activity of the PPAR γ 2 promoter. (A) NIH-3T3 cells were transiently transfected using FuGENE (Roche) with the PPAR γ 2 promoter (-609 to +52)/luciferase reporter construct and with a plasmid expressing C/EBP α to increase levels of reporter expression. Also, cells were transfected with the SV40/ β -galactosidase vector to control for transfection efficiency. Cells were treated with IFN γ for 2 h and 24 h before harvesting. Cell lysates were analyzed for luciferase and β -galactosidase activity. Relative light units (RLU) were determined by dividing luciferase activity by β -galactosidase activity. Results are shown as \pm standard deviation. (B) 3T3-L1 cells were treated with the MDI cocktail for 48 h and then were placed in DMEM with 10% FBS for 8 h. The cells were transiently transfected with the PPAR γ 2 promoter (-609 to +52)/luciferase reporter and SV40/ β -galactosidase constructs. The cells were then treated with IFN γ for 2 or 24 h. RLU was determined as described above. (C) 3T3-L1 preadipocytes were transiently transfected with the PPAR γ 2 promoter (-609 to +52)/luciferase reporter or the mutant PPAR γ 2 m217/212 (-609 to +52)/luciferase reporter and the SV40/ β -galactosidase vector. Cells were untreated (-) or treated (+) with IFN γ for 2 h. RLU was determined as described above.

or the mutant PPAR γ 2 m217/212 promoter (-609 to +52)/luciferase construct which was altered at two positions within the potential STAT1 binding site. A two hour treatment with IFN γ decreased luciferase activity by 40% in cells transfected with the wild type construct, as shown in Fig 3.4C. However, IFN γ treatment did not decrease the luciferase activity in cells transfected with the mutant PPAR γ 2 m217/212 construct (Fig 3.4C), but IFN γ treatment slightly increased luciferase activity. Furthermore, the basal level of luciferase activity of the mutant construct was lower, indicating that the nucleotides that were mutated (217/212) may contribute to the basal activity of the promoter.

These results suggested that modulation of PPAR γ transcription by IFN γ could be mediated by bases -221 to -207 of the PPAR γ 2 promoter, functioning as a STAT1 binding site. Since some genes have been shown to be regulated by STAT1/STAT3 heterodimers, we examined the ability of LIF, a potent activator of STAT3 in adipocytes (Stephens *et al.*, 1998), to induce binding to the IFN γ -sensitive element that we identified in the PPAR γ 2 promoter. As shown in Fig 3.5A, treatment with LIF resulted in two mobility shifts that were effectively competed with unlabeled probe. The intensity of these bands was substantially less than what we observed following IFN γ treatment (Fig 3.1). Considerably less of the labeled probe was bound by protein from LIF-treated extracts when compared to the amount of protein bound to the probe from IFN γ treated extracts (data not shown). To determine if the protein-binding complex contained STATs, the nuclear extracts were incubated with antibodies against STATs 1, 3, or 5A. Similar to IFN γ treatment, the STAT1 antibody was able to displace the LIF-regulated protein binding to the -221 to -207 oligonucleotide (Fig 3.5B). However, the band that was supershifted is very faint. Neither STAT3 nor STAT5A antibodies were capable of supershifting this interaction. Hence, only STAT1 was capable of binding to this site of the PPAR γ 2 promoter

following LIF stimulation but with substantially less binding to this site than that induced by IFN γ , presumably due to the greatly reduced nuclear levels of activated STAT1. Therefore, we examined the ability of LIF to regulate PPAR γ mRNA levels. Fully differentiated 3T3-L1

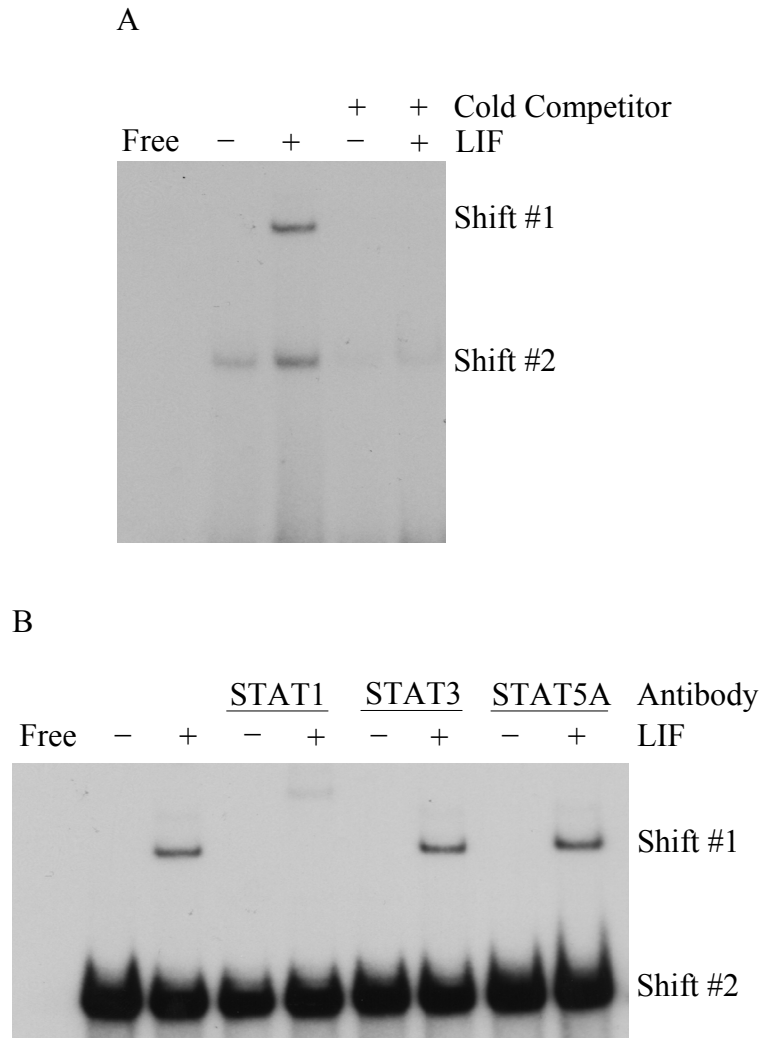


Figure 3.5 LIF-activated STAT1 binds the PPAR γ 2 promoter *in vitro*. (A) EMSA was performed with nuclear extracts prepared from 3T3-L1 adipocytes untreated (-) or treated (+) with LIF for 15 min. Nuclear extracts were incubated with the 32 P-labeled -221 to -207 oligonucleotide. Cold competition was performed by preincubating the nuclear extracts with the unlabeled oligonucleotide (8.9 μ M). (B) Nuclear extracts from LIF-treated 3T3-L1 adipocytes were preincubated with antibodies (4 μ g) for STATs 1, 3, or 5A prior to incubation with the 32 P-labeled -221 to -207 oligonucleotide. Protein-DNA complexes were resolved by EMSA. This is a representative experiment independently performed three times.

adipocytes were treated with either LIF or IFN γ and the expression of PPAR γ mRNA levels was examined by Northern blot analysis. As previously demonstrated, IFN γ treatment results in a substantial decrease in PPAR γ mRNA (Fig 3.6). Yet, exposure to LIF did not cause any striking changes in PPAR γ levels (Fig 3.6). In summary, these results suggest that the IFN γ -mediated repression of PPAR γ transcription is modulated by STAT1 and not by STAT3 in adipocytes. Moreover, LIF, an activator of STAT3 had no effect on PPAR γ mRNA accumulation.

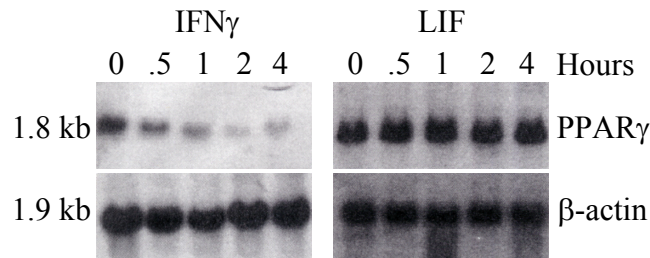


Figure 3.6 Acute LIF treatment does not affect the levels of PPAR γ mRNA in adipocytes. Total mRNA was isolated from fully differentiated 3T3-L1 adipocytes following treatment for various times with IFN γ or LIF, as indicated. Twenty micrograms of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis. β -Actin indicates that similar amounts of RNA were loaded for each sample. This is a representative experiment independently performed two times.

Our recent studies demonstrated that treatment of 3T3-L1 adipocytes with CT-1 resulted in a transient downregulation of PPAR γ mRNA (Zvonic *et al.*, 2004). Since CT-1 activates STAT1 in adipocytes, we investigated the ability of CT-1-activated STAT proteins to bind the -221 to -207 element of the PPAR γ 2 promoter. Therefore EMSAs were performed with nuclear extracts from 3T3-L1 adipocytes acutely treated with CT-1 for 15 minutes. As shown in Fig 3.7A two protein complexes that shifted the -221 to -207 oligonucleotide were detected, but only the slower-migrating protein complex was induced with CT-1 treatment in a dose-dependent manner. In order to determine whether the CT-1-induced protein complex contained STAT

proteins, supershift analysis was performed, using antibodies for STATs 1, 3, and 5A because these are the STATs activated by CT-1 in adipocytes. The STAT1 antibody fully supershifted the CT-1 induced protein complex; however the STAT3 and STAT5A antibodies had no effect on the mobility of the protein complex, as shown in Fig 3.7B, indicating that the protein complex may contain a STAT1 homodimer. Specificity of binding to the labeled oligonucleotide is shown in Fig 3.7C. Binding of the CT-1-activated protein complex to the radiolabeled -221 to -207 probe was competed with an excess of the unlabeled wild type oligonucleotide but not with a mutant oligonucleotide, nor with the -107 to -95 or the -431 to -408 oligonucleotides from the PPAR γ 2 promoter. To assess the importance of this STAT1 site in the CT-1-induced modulation of PPAR γ , we transiently transfected 3T3-L1 preadipocytes with the wild type PPAR γ 2 promoter (-609 to +52)/luciferase construct or with the mutant PPAR γ 2 m217/212 (-609 to +52)/luciferase construct. The results in Fig 3.7D demonstrate that a six-hour treatment with CT-1 resulted in a 30% decrease in luciferase activity of the wild type construct. However, the basal luciferase activity in cells transfected with the mutant PPAR γ 2 promoter construct was lower and was moderately increased following CT-1 treatment, indicating that the mutated bases within the STAT1 binding site are critical for the CT-1-induced downregulation of PPAR γ .

3.3 Discussion

Each STAT family member shows a distinct pattern of activation by cytokines and upon nuclear translocation, can regulate the transcription of particular genes in cell or tissue specific manners (Darnell, 1997). Although adipocytes express STAT proteins and the expression of three STAT family members is regulated during adipogenesis (Stephens *et al.*, 1996), this is the first study to identify an adipocyte target gene of activated STATs. In this investigation, we have identified PPAR γ 2 as a negatively regulated gene target of STAT1. We demonstrated that

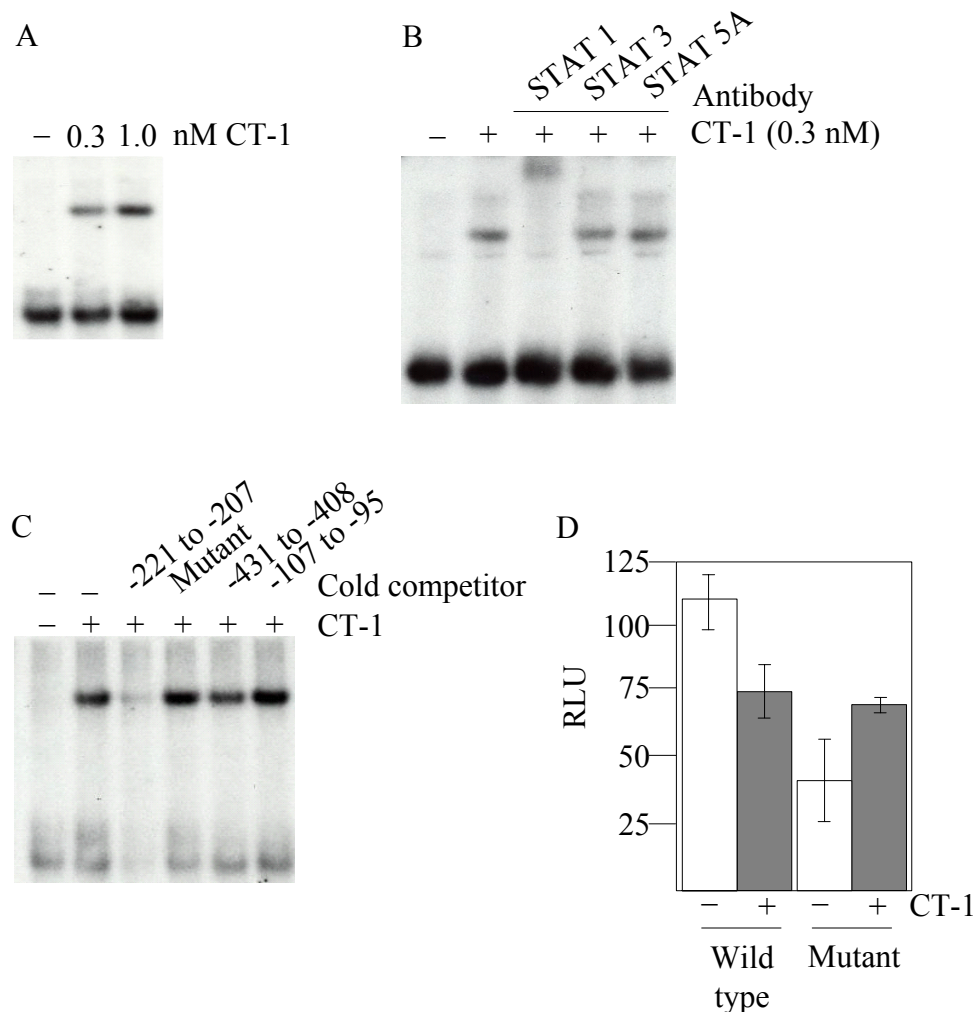


Figure 3.7 CT-1 induces STAT1 binding to the -221 to -207 site of the PPAR γ 2 promoter *in vitro*. (A) Nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) with the indicated dose of CT-1 for 15 min. The extracts were incubated with the 32 P-labeled -221 to -207 oligonucleotide for EMSA. (B) Nuclear extracts from CT-1-treated (0.3 nM) 3T3-L1 adipocytes were preincubated with antibodies for STATs 1, 3, or 5A for supershift analysis. The extracts were then incubated with the radiolabeled -221 to -207 probe. (C) Prior to incubation with the 32 P-labeled -221 to -207 oligonucleotide, nuclear extracts from CT-1-treated 3T3-L1 adipocytes were preincubated with unlabeled oligonucleotides of the wild type and mutant -221 to -207 site, and the -431 to -408 or the -107 to -95 sites from the PPAR γ 2 promoter. (D) 3T3-L1 preadipocytes were transiently transfected using Polyfect (Qiagen) with either the wild type PPAR γ 2 promoter (-609 to +52)/luciferase or the mutant PPAR γ 2 m217/212 (-609 to +52)/luciferase reporter constructs, along with the TK/renilla construct to control for transfection efficiency. After 48 hours, cells were untreated (-) or treated (+) with CT-1 for 6 h and then harvested for analysis. Relative light units (RLU) were calculated by dividing luciferase activity by renilla luciferase activity. Results are shown as +/- standard deviation.

nuclear STAT1 was capable of specifically binding to the -221 to -207 sequence in the PPAR γ 2 promoter following stimulation by IFN γ , LIF, and CT-1. This interaction correlates with the transcriptional repression of PPAR γ 2 by IFN γ and CT-1 that we previously reported (Waite *et al.*, 2001; Zvonic *et al.*, 2004). Moreover, transfection of two different cell types with a PPAR γ 2 reporter construct that contains this STAT1 binding site was negatively regulated by IFN γ but not when the site was mutated. These data strongly support our hypothesis that the IFN γ - and CT-1-induced repression of PPAR γ synthesis is mediated by STAT1. However, other studies suggest that it is possible that other STAT1 binding sites are present in the PPAR γ 2 promoter. For example, the p21 promoter has four distinct STAT1 binding sites (Chin *et al.*, 1996). Interestingly, we did not observe any effect of LIF, on PPAR γ mRNA accumulation. The activities of many promoters have been shown to be regulated by STAT1/STAT3 heterodimers. However, the STAT1 site we identified in the PPAR γ 2 promoter is likely bound by a STAT1 homodimer. This is supported by our data demonstrating that LIF treatment only results in weak binding to the PPAR γ promoter and supershift analysis which clearly indicates that STATs 3 and 5A are not part of the DNA-binding complex. LIF, a gp130 cytokine, potently activates STAT3 but weakly activates STAT1 (Stephens *et al.*, 1998) and does not regulate the expression of PPAR γ . Therefore, less STAT1 may be available in the nucleus to regulate transcription. Yet, treatment with CT-1, another gp130 cytokine, resulted in a transient downregulation of PPAR γ expression. Thus, while activation of STAT1 may be a molecular event which results in decreased PPAR γ expression, it is also possible that other signaling cascades regulate the ability of STAT1 to modulate PPAR γ .

Although STAT1 was originally identified as an activator of transcription, STAT1 has also been found to act as a negative regulator of several genes in other cell types (Sharma *et al.*, 1998; Ikeda *et al.*, 1999; Ramana *et al.*, 2000). The negative transcriptional regulation can occur by two mechanisms. First, STAT1 may activate the transcription of a second transcription factor that acts as a negative regulator. Second, STAT1 could directly bind to the promoter of the target gene and inhibit transcription. Our findings strongly suggest that the rapid IFN γ -induced repression of PPAR γ transcription and subsequent decrease in PPAR γ mRNA is due to a direct interaction of STAT1 with the PPAR γ 2 promoter. However, the IFN γ -induced decrease in PPAR γ transcription could be mediated in a STAT independent manner. Studies in STAT1 null cells have demonstrated that some of the actions of IFN γ are independent of STAT1 (Gil *et al.*, 2001; Ramana *et al.*, 2001). However, our observed regulation of PPAR γ tightly correlates with the presence of STAT1 in the nucleus and we have identified a highly specific STAT1 binding site by utilizing EMSA analysis. In summary, these results strongly suggest that STAT1 acts as a repressor of PPAR γ 2 transcription.

The quantity and activity of PPAR γ in adipose tissue contributes to insulin sensitivity. The PPAR γ +/- mouse model is resistant to insulin resistance and to diet-induced obesity (Miles *et al.*, 2000). These findings suggest that protection from diseases such as type II diabetes and/or obesity may be attained by the homeostatic balance of PPAR γ expression and activity. One mechanism of regulating PPAR γ expression is via proteasomal mediated degradation that is induced by both TZDs (Hauser *et al.*, 2000) and IFN γ (Waite *et al.*, 2001). However, PPAR γ expression can also be affected by changes in transcription and/or mRNA levels, which have been shown to be regulated by TNF α , TZDs, and IFN γ (Rosenbaum & Greenberg, 1998; Waite *et al.*, 2001). Interestingly, the IFN γ -CT-1 sensitive site we identified in the PPAR γ 2 promoter

is not present in the PPAR γ 1 promoter. However, treatment with IFN γ results in a decrease in both γ 1 and γ 2 proteins (Waite *et al.*, 2001). Yet, the decay of the γ 1 protein is much more rapid than the decay of the γ 2 protein. These results suggest that the regulation of PPAR γ transcription and degradation can be isoform specific. Although it is possible that the γ 1 promoter is also sensitive to transcriptional repression by IFN γ , we have been unsuccessful in our attempts to identify an IFN γ -sensitive element in the γ 1 promoter. To date, there is minimal information about transcription factors that bind to the PPAR γ promoters. A site that binds C/EBP α and C/EBP δ has been identified in the PPAR γ 2 promoter that positively regulates PPAR γ expression (Clarke *et al.*, 1997). In addition, the PPAR γ 1 and γ 3 promoters both contain a consensus E-box motif that has been shown to mediate the regulation of the PPAR γ gene by ADD-1/SREBP-1 and SREBP-2 (Fajas *et al.*, 1999). Our studies have shown that the γ 2 promoter contains a STAT1 binding site that likely mediates the IFN γ - and CT-1-induced repression of PPAR γ 2 transcription. In conclusion, there is very little known about the regulation of the PPAR γ promoters. The modulation of PPAR γ synthesis by IFN γ , CT-1 and other cytokines may lead to insights into the molecular mechanisms regulating energy homeostasis and may contribute to understanding the defects underlying obesity and NIDDM.

CHAPTER 4: STAT1 BINDS TO THE LIPOPROTEIN LIPASE PROMOTER *IN VITRO**

4.1 Introduction

Lipoprotein lipase (LPL) is an enzyme which catalyzes the hydrolysis of serum triglycerides from very low density lipoproteins and chylomicrons for uptake of free fatty acids and lipid storage in adipose tissue (Goldberg, 1996). Interferon- γ (IFN γ) has been shown to decrease the expression and activity of LPL in cultured mouse and rat preadipocytes (Gregoire *et al.*, 1992) and in the 3T3-F442A adipocyte cell line (Feingold *et al.*, 1992; Doerrler *et al.*, 1994). Since IFN γ activates the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway in adipocytes, resulting in phosphorylation and nuclear translocation of STATs 1 and 3, we hypothesized that STATs directly regulated the expression of LPL in adipocytes. In this study we have identified a STAT binding site in the promoter of the murine LPL gene. Furthermore, we have demonstrated the binding of IFN γ -activated STAT1 to this site. Our results suggest that STAT1 has a role in the regulation of LPL expression to modulate rate of lipolysis in adipocytes.

4.2 Results

Previous studies have demonstrated that IFN γ treatment inhibits adipocyte differentiation and decreases expression of LPL in adipocytes (Keay and Grossberg, 1980; Grossberg and Keay, 1980; Gregoire *et al.*, 1992; Doerrler *et al.*, 1994). Therefore, we examined the murine LPL promoter sequence (GenBank M63335) for potential STAT binding sites. Our analysis revealed two putative elements in the LPL promoter, shown in Table 4.1, which resembled the STAT

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consensus IFN γ -activated sequence (GAS)/STAT induced element (SIE) sequence (TTCNNNGAA).

Table 4.1 STAT1 binding sites in the LPL promoter

Gene	Position	Sequence	IFN γ Responsive
Consensus GAS/SIE		TTC NNN GAA	
LPL	-125 to -108	CGT TTG CTC AAA TAT TT	No
LPL	-938 to -921	TG CTT TTC TAC AAA CAT G	Yes
LPL	mutant -938	TG CTT CTC TAC CAA CAT G	No

To analyze the binding of protein complexes to oligonucleotides corresponding to these sequences of the LPL promoter, electrophoretic mobility shift assays (EMSAs) were performed with nuclear extracts isolated from 3T3-L1 adipocytes that were unstimulated or treated for 15 min with IFN γ . As shown in Fig. 4.1A, the -938 to -921 site of the LPL promoter was bound by two IFN γ -sensitive protein complexes indicated by the arrows. The binding of nuclear extracts to the radiolabeled -938 to -921 site was successively competed with increasing concentrations (7pg/ μ l to 40ng/ μ l) of cold competitor (Fig. 4.1A). Additional specificity of this interaction is shown in Fig. 4.1B. The IFN γ -induced protein binding to a labeled oligonucleotide (-938 to -921) of the LPL promoter (Lane 3) was effectively competed with an unlabeled wild-type oligonucleotide (Lane 4), but not a mutant oligonucleotide (Lane 5). Interestingly, binding at this site was also effectively competed with the STAT1 binding site that we previously identified in the PPAR γ 2 promoter (Lane 6) (Hogan and Stephens, 2001). The other GAS-like element we identified in the LPL promoter at -125 to -108 was unresponsive to IFN γ treatment (data not shown).

As shown in the EMSA analysis in Fig 4.2A, the IFN γ -sensitive site was bound by nuclear proteins. We did not observe binding by cytosolic proteins, but the lack of detectable binding may be attributed to the apparent degradation of the probe, as less unbound probe

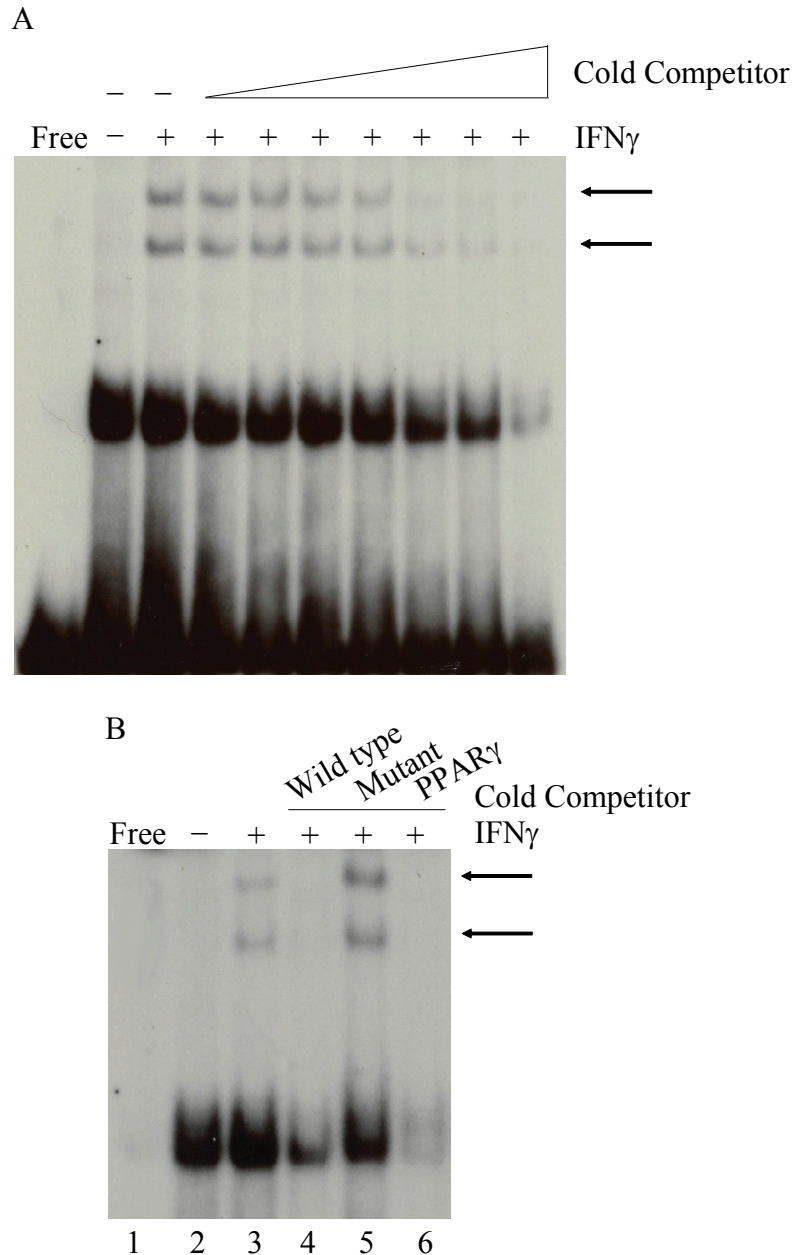


Figure 4.1 The identification of a specific IFN γ -sensitive binding site in the LPL promoter. (A) Nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) for 15 min with IFN γ . The extracts were preincubated with increasing concentrations of unlabeled -938 to -921 oligonucleotide (7 pg/ μ l to 40 ng/ μ l). The nuclear extracts were then incubated with the labeled probe and subjected to EMSA. (B) Nuclear extracts from untreated (-) or IFN γ -treated (+) 3T3-L1 adipocytes were preincubated with unlabeled oligonucleotides of the -938 to -921 site of the LPL promoter, a mutant of the -938 to -921 site, or an oligonucleotide from the PPAR γ 2 promoter which binds STAT1 proteins present in nuclear extracts of IFN γ -stimulated 3T3-L1 adipocytes. The nuclear extracts were then incubated with the labeled -938 to -921 probe and subjected to EMSA.

appeared at the bottom of the gel in lanes containing cytosolic protein than in lanes containing nuclear protein. To determine if STAT1 was a constituent of the bound complexes, we performed supershift analysis with a STAT1 antibody. Both of the IFN γ -sensitive protein complexes were completely supershifted by the STAT1 antibody, as shown in Fig. 4.2A. We also examined the effects of GH and LIF on the ability to induce binding to this site within the LPL promoter. Our previous results demonstrate that LIF treatment can also result in STAT1 activation in 3T3-L1 adipocytes (Balhoff and Stephens, 1998) and GH can activate STAT5 proteins (Stephens *et al.*, 1998). As shown in Fig. 4.2B, these results demonstrate that only IFN γ , and not LIF or GH, can result in binding to the -938 to -921 site of the LPL promoter.

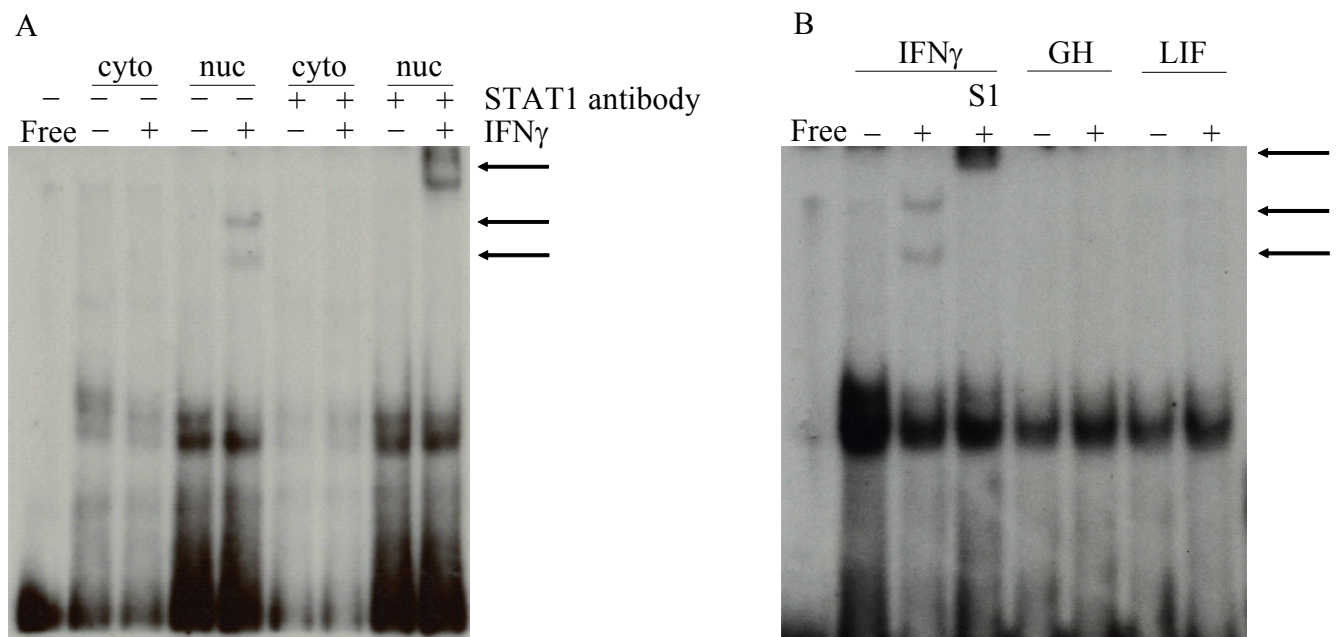


Figure 4.2 IFN γ -activated STAT1 binds to the LPL promoter *in vitro*. (A) Cytosolic and nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated (-) or treated (+) with IFN γ for 15 min. The extracts were incubated with 32 P-labeled -938 to -921 oligonucleotide for EMSA analysis. For supershift analysis, cytosolic and nuclear extracts were preincubated with a STAT1 antibody. (B) Nuclear extracts from 3T3-L1 adipocytes, untreated (-) or treated (+) with IFN γ , GH, or LIF, were incubated with the labeled -938 to -921 probe. Supershift analysis was performed by preincubating the nuclear extracts with the STAT1 antibody (indicated as S1).

Previous studies have clearly demonstrated that IFN γ treatment can result in an inhibition of LPL transcription, mRNA and enzymatic activity. Our results demonstrate that IFN γ -activated STAT1 can bind to the LPL promoter *in vitro* and may play a role in the repression of LPL transcription. Hence, we investigated the ability of IFN γ to regulate LPL protein expression. As shown in Fig. 4.3, IFN γ treatment of fully differentiated 3T3-L1 adipocytes resulted in a notable decrease in LPL protein levels following 48 h of treatment.

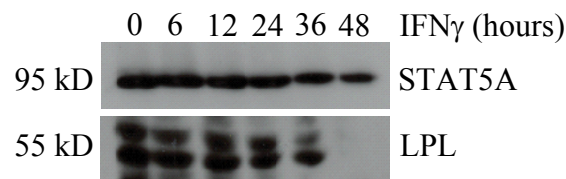


Figure 4.3 IFN γ treatment decreases LPL protein levels. Whole cell extracts were prepared from 3T3-L1 adipocytes following treatment with IFN γ for the various times indicated in the figure. One hundred micrograms of each sample was resolved by SDS-PAGE and analyzed by Western blot. The detection system was horseradish peroxidase and enhanced chemiluminescence (Pierce).

4.3 Discussion

Although IFN γ is primarily known for its roles in immunological responses, it has been shown to have potent effects on adipocyte gene expression (Doerrler *et al.*, 1994; Waite *et al.*, 2001) and to inhibit the differentiation of cultured rodent preadipocytes (Gregoire *et al.*, 1992). In adipocytes, IFN γ treatment results in decreased LPL activity and increased lipolysis (Doerrler *et al.*, 1994). In 3T3-F442A adipocytes, exposure to IFN γ results in decreased LPL and FAS expression. Recent studies in macrophages have shown that IFN γ can inhibit LPL at the transcriptional level (Hughes *et al.*, 2002). Hence, the goal of our project was to identify IFN γ -sensitive sites within the LPL promoter.

Our studies identified a region of the LPL promoter that binds two protein complexes in an IFN γ -dependent manner. The specificity of binding to this site was demonstrated by the use of cold competitor and mutant oligonucleotides (Fig. 4.1). Interestingly, binding to this site within the LPL promoter was effectively competed with a STAT1 binding site that we previously identified in the PPAR γ 2 promoter (Hogan and Stephens, 2001). Our previous studies demonstrated that STAT1, but not STATs 3 or 5A, could bind to the -221 to -207 site of the PPAR γ 2 promoter (Hogan and Stephens, 2001). These results suggested that both IFN γ -sensitive protein complexes that bind the LPL promoter *in vitro* contained phosphorylated STAT1 proteins. This hypothesis is supported by our data demonstrating that the IFN γ -sensitive element in the LPL promoter specifically binds nuclear, but not cytosolic, extracts isolated from IFN γ -treated 3T3-L1 adipocytes (Fig. 4.2A). These results are further confirmed by supershift analysis with STAT1 antibodies which revealed that both of these complexes contained STAT 1 proteins (Fig. 4.2). However, at this time, we do not know what proteins account for the difference between the two IFN γ -sensitive STAT1 protein complexes that bind to the -938 to -921 region of the LPL promoter. We hypothesize that other adipocyte expressed STATs, in particular, STAT3 and STAT5, are not part of these binding complexes. This hypothesis is supported by our results which demonstrate that LIF, a potent STAT3 activator and GH, a potent STAT5 activator, do not confer binding to this site (Fig. 4.2B). In addition, this effect appears to be specific for IFN γ , since LIF treatment, which induces STAT1 phosphorylation and nuclear translocation in these cells (Stephens *et al.*, 1998), did not confer binding to this site. We predict that the IFN γ -induced STAT1 binding to the LPL promoter plays a role in the IFN γ -mediated repression of LPL transcription that has recently been observed (Hughes *et al.*, 2002). In addition, IFN γ has been previously shown to inhibit LPL mRNA and LPL activity. Our results

demonstrating that IFN γ induced a decrease in LPL protein expression (Fig. 4.3) support these studies. In summary, we have identified a STAT1 binding site within the murine LPL promoter which likely plays a role in the IFN γ -induced decrease in LPL expression.

CHAPTER 5: REGULATION OF FATTY ACID SYNTHASE BY STAT5

5.1 Introduction

Fatty acid synthase (FAS) is the key enzyme in *de novo* lipogenesis, catalyzing the reactions for the synthesis of palmitate (Sul and Wang, 1998). FAS is regulated primarily at the level of transcription and is sensitive to nutritional and hormonal regulation (Sul and Wang, 1998). Previous studies have demonstrated that growth hormone (GH), a cytokine which activates signal transducer and activator of transcription 5 (STAT5) in adipocytes (Balhoff and Stephens, 1998), abrogates the induction of FAS expression by insulin and down-regulates basal expression of FAS in 3T3-F442A cells (Yin *et al.*, 2001a). Although insulin-regulated regions of the FAS promoter have been extensively studied, there has not been a conclusive study to characterize a GH-responsive region or to determine the potential role of STATs in the modulation of FAS expression. Since GH and prolactin (PRL) are potent activators of STAT5 (Balhoff and Stephens, 1998; Nanbu-Wakao *et al.*, 2000), we hypothesized that STAT5 directly regulates the expression of FAS. In this study we have demonstrated that GH and PRL treatment of 3T3-L1 adipocytes resulted in a decrease of FAS mRNA and protein. In addition, we have identified a region within the FAS promoter that is responsive to PRL. This region contains a non-consensus STAT5 binding site, which, when mutated, results in a loss of sensitivity to PRL. Our results clearly demonstrate that STAT5A binds to this non-consensus sequence and strongly suggest that FAS is a direct target for regulation by STAT5. These data suggest a novel means for regulation of FAS expression in adipocytes and reveal a mechanism by which STAT5 proteins and PRL exert anti-adipogenic effects.

5.2 Results

GH is known to reduce adipose tissue *in vivo* (Magri *et al.*, 1990) and decrease the expression of FAS in adipocytes (Yin *et al.*, 1998). GH is also a potent activator of STAT5 in

adipocytes (Balhoff and Stephens, 1998). Hence we hypothesized that STAT5 proteins directly modulate FAS expression. Therefore, we examined the regulation of FAS by two STAT5 activators, GH and PRL. As shown in Fig. 5.1A, we observed that both GH and PRL resulted in a decrease in FAS mRNA in fully differentiated 3T3-L1 adipocytes. GH decreased FAS mRNA by 12 hours of treatment, and PRL treatment resulted in decreased expression within 6 hours. In an independent experiment we observed that both GH and PRL also resulted in a decrease in expression of FAS protein levels (Fig. 5.1B). To demonstrate the specificity of this effect, fully differentiated 3T3-L1 adipocytes were treated with PRL for 8 hours with the various doses indicated in Fig. 5.1C. There was a notable decrease in FAS protein level with 8.8 nM PRL treatment, and the decrease of FAS protein levels by PRL treatment was dose-dependent. An acute treatment of 1.3 μ M PRL was included as a positive control for STAT5 phosphorylation. The level of STAT5A expression was unchanged by GH or PRL (Fig. 5.1B and 5.1C) and is shown to indicate even loading of protein samples.

Clearly, the analysis of mRNA and whole cell extracts demonstrated that activators of STAT5 decreased expression of FAS protein and mRNA. Yet, it was unclear if the effects of GH and PRL were mediated by affecting FAS transcription and/or protein turnover. To assess whether the effects of PRL on FAS expression could be attributed to changes in the turnover of FAS protein, we examined the loss of FAS in the presence of cycloheximide (5 μ M) or ethanol, a vehicle control. Whole cell extracts were collected at various times and used for Western blot analysis. As shown in Fig. 5.2, either cycloheximide or prolactin treatment caused a decrease in FAS protein. In the presence of cycloheximide, a loss of FAS was observed at 8 hours, regardless of the presence of PRL. PRL treatment alone decreased the level of FAS within 12 hours of treatment. These results indicate that PRL does not affect the turnover of FAS protein

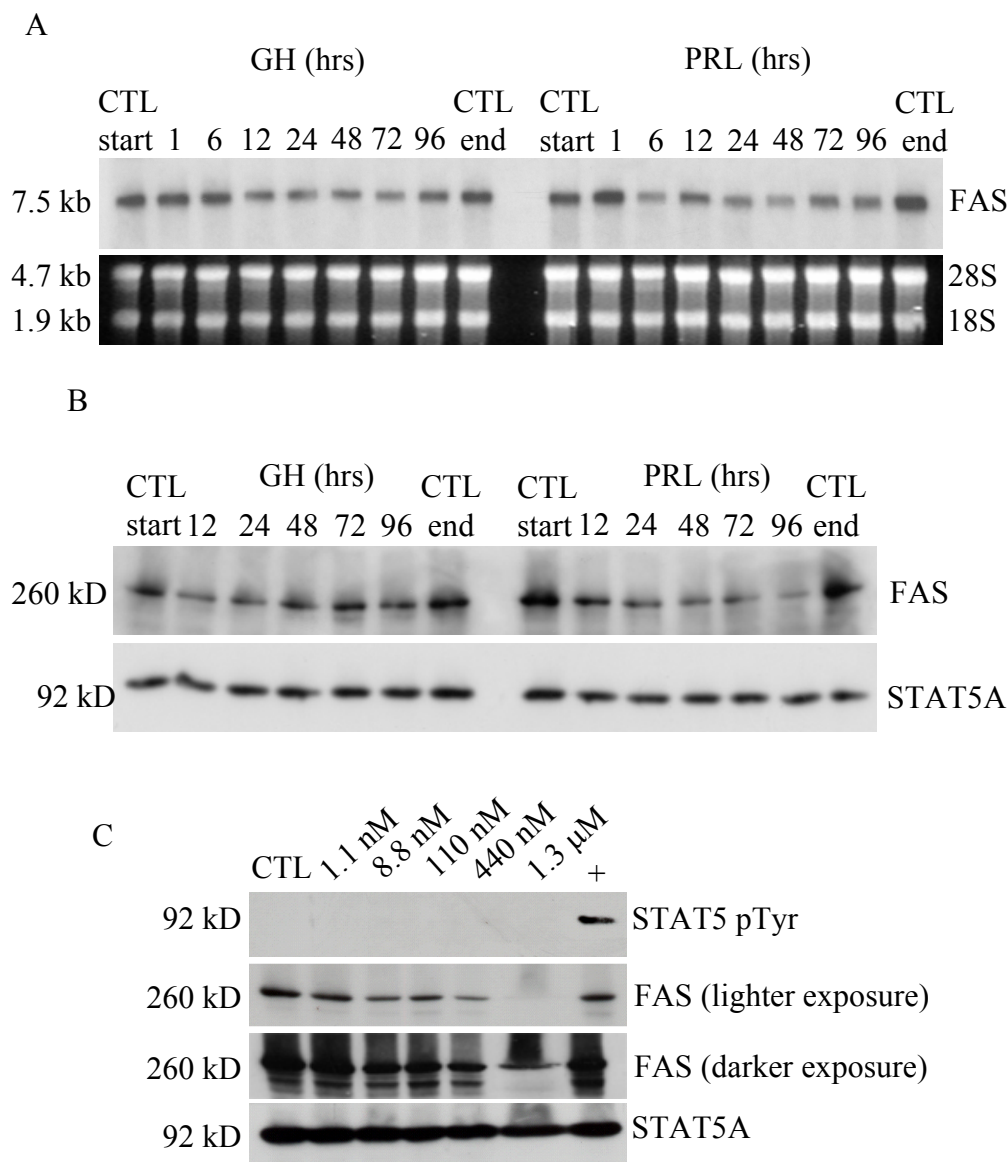


Figure 5.1 Activators of STAT5 decrease expression of FAS in 3T3-L1 adipocytes. (A) Total RNA was isolated from 3T3-L1 adipocytes following treatment with GH (11.3 nM) or PRL (1.4 μ M) for the times indicated in hours. Untreated cells (CTL) were harvested at the start and end of the time course. Fifteen μ g of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis with radiolabeled probe for FAS. Ethidium bromide staining of 28S and 18S RNA is included as a loading control. This is a representative experiment independently performed two times. (B) Mature 3T3-L1 adipocytes were stimulated with GH (11.3 nM) or PRL (1.4 μ M) for the times indicated. One hundred μ g of protein from whole cell extracts were loaded into the gel for each sample. The samples were subjected to SDS-PAGE, and then were transferred to nitrocellulose for immunoblot analysis. This is a representative experiment independently performed two times. (C) Mature 3T3-L1 adipocytes were stimulated with PRL with the doses indicated for 8 hr. Western analysis was performed as described above. This is a representative experiment independently performed two times.

in 3T3-L1 adipocytes and suggest that PRL may exert its effects on FAS at the levels of transcription.

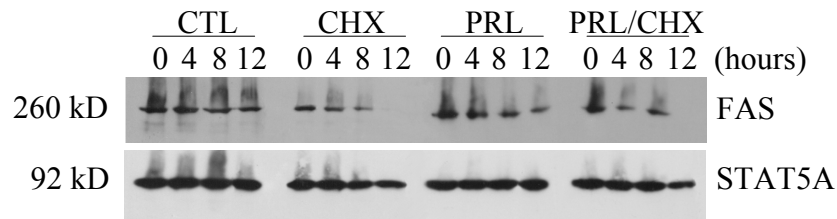


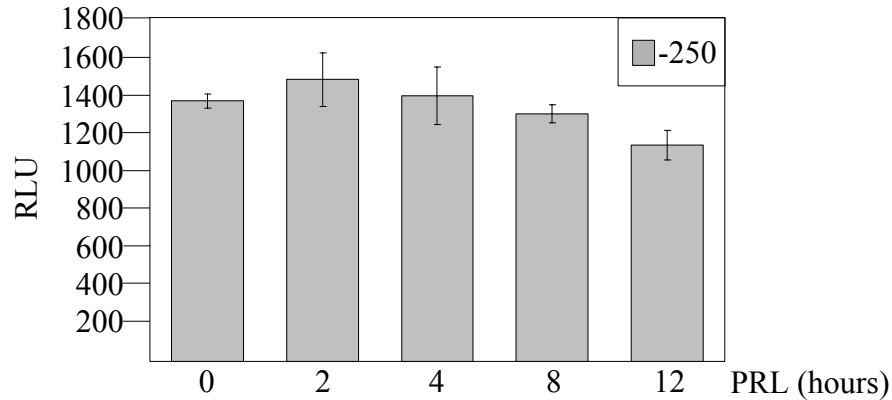
Figure 5.2 PRL does not affect turnover of FAS protein. Fully differentiated 3T3-L1 adipocytes were treated with cycloheximide (CHX, 5 μ M) and PRL (1.4 μ M) for the times indicated. One hundred μ g of protein from whole cell extracts were loaded into the gel for each sample. The samples were subjected to SDS-PAGE, and then were transferred to nitrocellulose for immunoblot analysis. This is a representative experiment independently performed two times.

Our data indicate that PRL may directly, transcriptionally regulate the expression of FAS. Recent studies by Yin and colleagues (2001a) have shown that GH abrogates the induction of FAS by insulin and suggested that the -112 to +65 region of the rat FAS promoter was sensitive to the regulation by GH. Therefore, we hypothesized that a STAT5 recognition element may be present in this region. Although the regulation of FAS by GH has been investigated, the mechanism of regulation by PRL in adipocytes has not been elucidated. To address these questions, 3T3-L1 preadipocytes were transiently transfected with a luciferase construct containing the FAS promoter fragment of -250 to +65. After 48 hours, cells were stimulated with PRL for the times indicated in Fig. 5.3A and then were analyzed for luciferase activity. We observed that PRL treatment had no significant effects on the relative luciferase activity (Fig. 5.3A), clearly demonstrating that the -250 to +65 region of the FAS promoter is not sensitive to PRL. Therefore, we examined the PRL responsiveness of two additional rat FAS promoter/luciferase constructs that incorporated larger regions, -1594 to +65 and -700 to +65, of the promoter. Transfection of these constructs into 3T3-L1 cells revealed a PRL-responsive

region present between -1594 and -700 of the rat FAS promoter. As shown in Fig. 5.3B, treatment with PRL resulted in a 64% decrease in luciferase activity of the rat FAS promoter (-1594 to +65)/luciferase construct. Although the basal level of luciferase activity for the rat FAS promoter (-700 to +65)/luciferase construct was similar to that of the -1594 to +65 construct, PRL had no effect on the level of luciferase activity (Fig. 5.3B). These data demonstrate that a PRL-sensitive region exists between -1594 and -700 of the FAS promoter.

Since PRL is a potent activator of STAT5, we hypothesized that the -1594 to -700 region of the FAS promoter may contain a STAT5 binding site conferring PRL responsiveness. Therefore, we examined the rat FAS promoter (GenBank X62889) for the presence of STAT consensus sites (TTCNNNGAA). An examination of the FAS promoter did not result in the identification of any sequences that precisely matched the STAT consensus site. However, as shown in Table 5.1, we identified four regions that were similar to the consensus sequence. To evaluate these potential STAT5 binding sites, we performed a series of electrophoretic mobility shift assays (EMSA). For these experiments, nuclear and cytosolic extracts were prepared from 3T3-L1 adipocytes that were acutely treated with PRL for 15 minutes. As shown in Fig. 5.4A, PRL did not induce the binding of nuclear protein complexes to the -951 to -933, -1226 to -1214, or -4639 to -4623 regions of the FAS promoter. The induction of binding by a PRL-activated protein complex to the rat β -casein STAT5 binding site (Schmitt-Ney *et al.*, 1991) is included as a positive control. However, we did observe PRL-dependent binding by a nuclear protein complex to the -908 to -893 region of the FAS promoter (Fig. 5.4B). In order to determine the specificity of binding, an EMSA was performed using a mutant version of the -908 to -893 oligonucleotide, in which two nucleotides were changed (Table 5.1). As shown in Fig. 5.4B, there was no detectable binding to the mutant form of the binding site following PRL

A



B

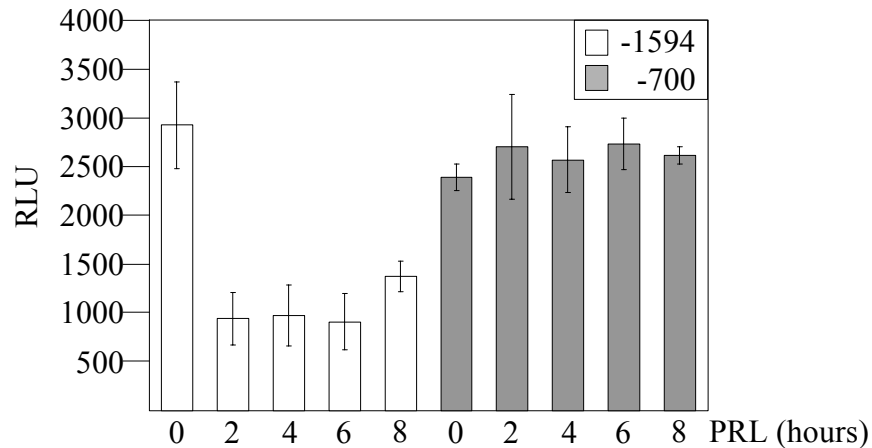


Figure 5.3 A PRL responsive region resides between -1594 and -700 of the rat FAS promoter. (A) Proliferating 3T3-L1 cells were transiently transfected with the rat FAS promoter (-250 to +65)/luciferase construct and the TK/renilla vector to control for transfection efficiency. After 48 hours of transfection, cells were stimulated with PRL (2.8 μ M) for the times indicated. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity. Results are shown as \pm standard deviation. (B) 3T3-L1 preadipocytes were transiently transfected with the (-1594 to +65) or (-700 to +65) rat FAS promoter/luciferase constructs. RLU was determined as described above.

stimulation. In addition, binding of the PRL-activated protein complex was successively competed with increasing concentrations of the unlabeled -908 to -893 oligonucleotide (Fig. 5.4C). Further evidence of specificity is shown in Fig. 5.4D, in which an excess of the unlabeled -908 to -893 oligonucleotide competed away binding induced by PRL treatment (Lane 4), whereas the mutant form of the oligonucleotide did not (Lane 5). Although we did not detect

binding to the -951 to -933 site, an excess of this oligonucleotide moderately diminished binding to the radiolabeled -908 to -893 probe (Lane 6). Yet, there was no appreciable competition of binding by the -1226 to -1214 oligonucleotide (Lane 7). As anticipated, binding was competed away with the STAT5 binding site of the rat β -casein promoter. Interestingly, a STAT3 binding site, -168 to -148 from the rat α_2 -macroglobulin promoter (Hattori *et al.*, 1990), which was included as a control, also resulted in binding competition. However, binding was not competed with a STAT 1 binding site that is present at -221 to -207 of the PPAR γ 2 promoter (Hogan and Stephens, 2001).

Table 5.1 Potential STAT5 binding sites in the FAS promoter

Gene	Position	Sequence	PRL Responsive
Consensus GAS/SIE		TTC NNN GAA	
FAS	-908 to -893	GGG TCC CGG AAA CCA G	Yes
FAS	mutant -908	GGG TCA AGG AAA CCA G	No
FAS	-951 to -931	C CCT TTC AAA AGA	No
FAS	-1226 to -1214	C TCC TTC CAC AGA GAG	No
FAS	-4639 to -4621	A ACT TTT TGA AAC	No
β -casein	-100 to -87	G GTT TTC TTG GAA TT	Yes

To determine if the protein complex binding the -908 to -893 contained STAT proteins, we performed supershift analysis using antibodies directed against the STAT proteins expressed in adipocytes. As shown in Fig. 5.5, the protein complex induced by PRL was fully supershifted with a STAT5A antibody (lane 5) and weakly supershifted with a STAT5B antibody (lane 6). STAT1 and STAT3 antibodies had no effect on the mobility of the complex (lanes 3 and 4). We also investigated the specificity of binding by STAT proteins by comparing the binding induced by LIF (0.5 nM), a cytokine which activates STAT1 and STAT3, but not STAT5 in 3T3-L1 adipocytes (Stephens *et al.*, 1998). LIF stimulated binding to the -908 to -893 site by a protein complex that exhibited highly reduced binding affinity and slightly faster mobility than the

complex induced by PRL (Fig. 5.5). Moreover, the LIF-induced complex was supershifted by a STAT1 antibody (lane 8), but not with antibodies for STAT3 or STAT5A (lanes 9 and 10).

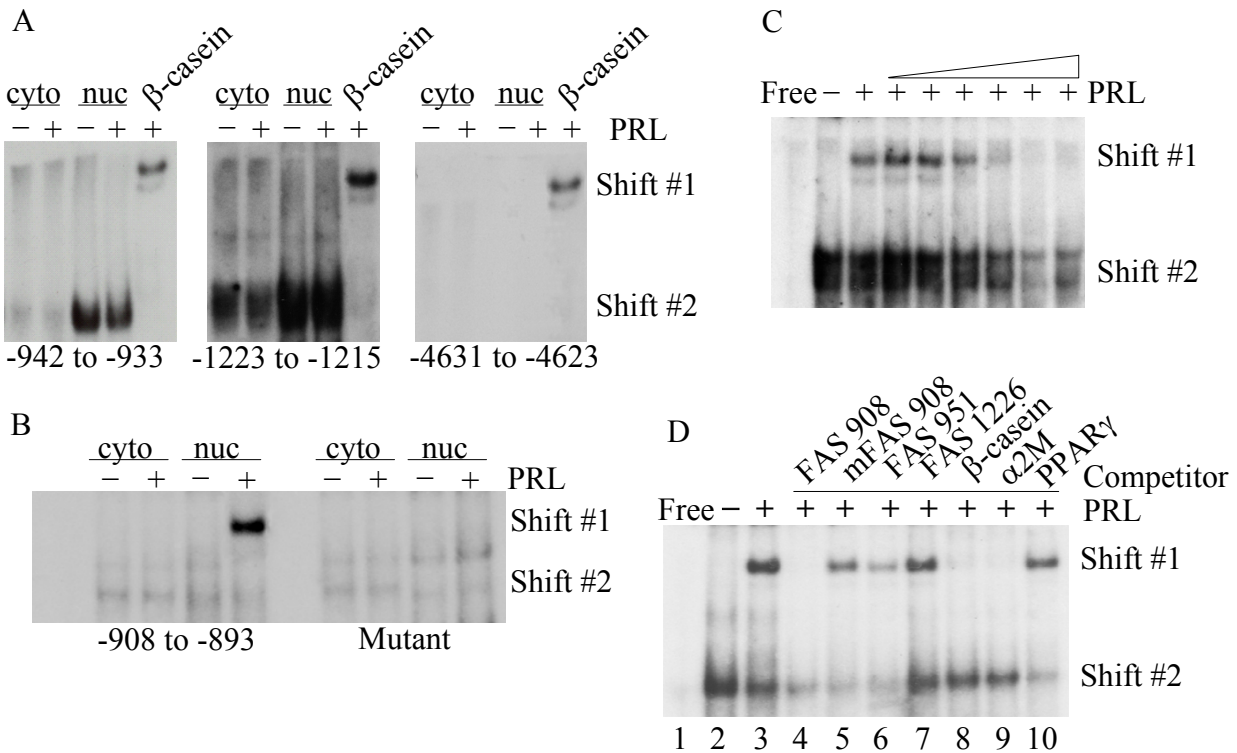


Figure 5.4 PRL stimulates binding of nuclear proteins to the -908 to -893 site in the rat FAS promoter. (A) Nuclear extracts were prepared from differentiated 3T3-L1 adipocytes that were untreated (-) or treated (+) with PRL (1.4 μ M) for 15 min. For each sample, 10 μ g of protein were incubated with 50,000 cpm/ml of the indicated 32 P-labeled probe of the FAS promoter. The protein-DNA complexes were resolved by EMSA. Binding to the rat β -casein (-101 to -87) site is included as a positive control. This is a representative experiment independently performed two times. (B) Binding to the -908 to -893 and the mutant oligonucleotides by PRL-induced protein complexes was analyzed as described above. (C) Successive cold competition was performed with 75 nM to 15 μ M of the unlabeled -908 to -893 oligonucleotide. EMSA was performed as described above. (D) Nuclear extracts were preincubated with an excess of the indicated unlabeled oligonucleotides (15 μ M). EMSA was performed as described above.

Our data clearly demonstrate that the -908 to -893 region of the rat FAS promoter binds nuclear PRL-activated STAT5 proteins *in vitro*. In order to determine if this region of the FAS promoter contributed to the regulation of FAS by PRL in living cells, we performed site-specific mutagenesis to alter two basepairs at positions -902 and -901 within the rat FAS promoter (-1594

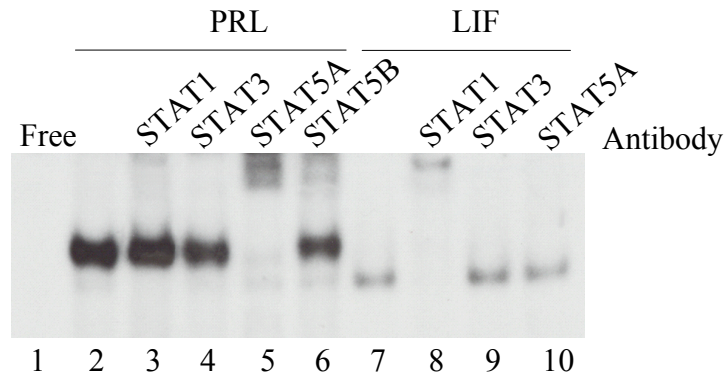


Figure 5.5 PRL induces STAT5A binding to the -908 to -893 site in the rat FAS promoter.

Nuclear extracts were prepared from 3T3-L1 adipocytes treated with PRL (1.4 μ M) or LIF (0.5 nM) for 15 min. For each sample, 10 μ g of protein were preincubated with 4 μ g of the indicated antibody and then incubated with 50,000 cpm/ml of the indicated 32 P-labeled probe of the -908 to -893 site in the FAS promoter. The protein-DNA complexes were resolved by EMSA. This is a representative experiment independently performed two times.

to +65)/luciferase construct. We have shown that this mutation abolished binding of PRL-induced proteins to this site (Fig. 5.4B and 5.4D). Transfection of the wild-type and mutant constructs into 3T3-L1 cells revealed that the basal level of luciferase activity was unaffected by mutation of the -902 and -901 basepairs of the FAS promoter (Fig. 5.6). However, the 60% decrease in luciferase activity induced by PRL for the wild-type construct was eliminated with the mutation. Thus, these data clearly indicate that the -908 to -893 site of the FAS promoter is sensitive to PRL and suggest that this site confers the negative regulation of FAS by PRL-activated STAT5A protein complexes.

5.3 Discussion

The novel data in this study include data demonstrating that FAS levels are decreased following stimulation with activators of STAT5 in 3T3-L1 adipocytes, the identification of a PRL-responsive region of the rat FAS promoter, and the characterization of a STAT5 binding site in this region. These findings strongly suggest that STAT5A directly represses the expression of FAS in adipocytes. Moreover, our data indicate that STAT5A has an anti-

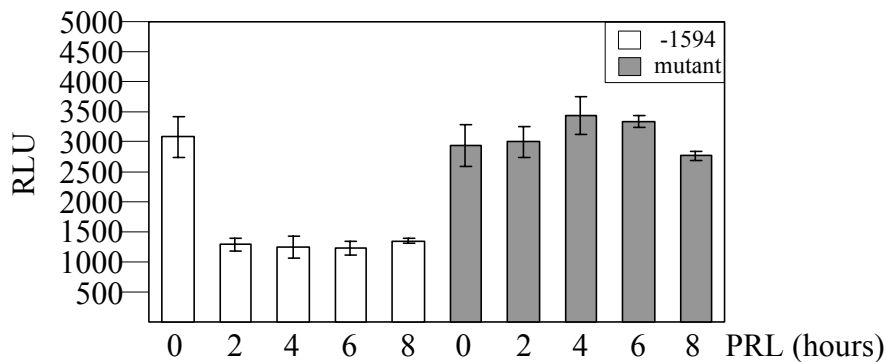


Figure 5.6 The -908 to -893 region of the rat FAS promoter confers sensitivity to PRL.

Proliferating 3T3-L1 cells were transiently transfected with the FAS (-1594 to +65)/luciferase reporter wild-type construct or with the FAS m901/902 (-1594 to +65)/luciferase mutant construct. Cells were also transfected with the TK/renilla construct to normalize for transfection variability. After 48 hours of transfection, cells were stimulated with PRL (2.8 μ M) for the times indicated. Relative light units (RLU) were calculated by dividing firefly luciferase activity by renilla luciferase activity. Results are shown as +/- standard deviation.

lipogenic function in mature adipocytes, in addition to an adipogenic role previously described by our laboratory (Floyd and Stephens, 2003) and others (Yarwood *et al.*, 1995; Teglund *et al.*, 1998; Shang *et al.*, 2003), indicating that the functions of STAT5A may depend on the developmental stage of the cells

Previous studies have shown that GH, an activator of STAT5, attenuated the induction of FAS in adipocytes by insulin in 3T3-F442A adipocytes (Yin *et al.*, 1998) and decreased expression of FAS *in vivo* (Magri *et al.*, 1990). We observed that two activators of STAT5 in adipocytes, GH and PRL, decreased protein and mRNA expression of FAS in 3T3-L1 adipocytes. These results are consistent with the findings that GH (Lanna *et al.*, 1995) and PRL (Flint *et al.*, 1981) inhibit the activity of FAS in adipose tissue. Our results indicate that PRL did not affect the protein turnover of FAS, but indicated that changes in FAS levels are mediated at the transcriptional level. Our experiments are supported by other studies which demonstrate that FAS can be modulated at the transcriptional level (Bennett *et al.*, 1995; Roder *et al.*, 1997;

Boizard *et al.*, 1998; Kim *et al.*, 1998; Sul and Wang, 1998; Yin *et al.*, 2001a). However, increased turnover of FAS mRNA in 3T3-F442A adipocytes has been demonstrated as one of the means through which GH attenuates the induction of FAS by insulin (Yin *et al.*, 1998). Nonetheless, our results strongly suggest that the PRL-induced repression of FAS is mediated by an inhibition of transcription.

In order to elucidate the mechanism of transcriptional regulation by PRL, we investigated the regulation of the FAS promoter. Although a previous study indicated that the -112 to +65 region of the rat FAS promoter was sensitive to regulation by GH (Yin *et al.*, 2001a), we did not observe PRL-mediated regulation of the rat FAS promoter within this region (Fig. 5.3A). However, our analysis of larger regions of the rat FAS promoter clearly indicated that a PRL-responsive region existed between -1594 to -700 of the rat FAS promoter (Fig. 5.3B). Yin and co-workers (2001a) have previously shown that GH attenuated the stimulation by insulin of the rat FAS promoter (-112 to +65)/luciferase construct, a region of the promoter which does not contain a STAT consensus site. Furthermore, in another study, it was demonstrated that staurosporine, an inhibitor of JAK/STAT signaling, did not block the effect of GH on insulin-stimulated FAS expression (Yin *et al.*, 2001b). Thus, it is unlikely that STAT5 proteins mediate the inhibitory effects of GH on insulin regulation of FAS. Yet, in light of our current findings, we postulate that the repression of basal levels of FAS by PRL is directly regulated by STAT5 proteins via binding to a site within the -1594 to -700 region of the rat FAS promoter.

Since PRL is a potent activator of STAT5 (Fig. 5.1B), we examined the rat FAS promoter for sites that resembled the STAT consensus sequence, TTCNNNGAA. We identified four sites that were similar to the STAT consensus, but our analysis by EMSA indicated that only one site, at position -908 to -893, was bound by a PRL-induced nuclear protein complex. We have also

shown that binding was specific (Fig. 5.4). In our experiments, the PRL-induced protein complex was fully supershifted by a STAT5A antibody (Fig. 5.5). The functional significance of the -908 to -893 site was determined by mutating two nucleotides at positions -902 and -901, within the STAT5 binding site of the rat FAS promoter (-1594 to +65)/luciferase construct. Mutation of this site completely abrogated the downregulation by PRL that was observed with the wild-type construct (Fig. 5.6). Taken together, these data strongly suggest that -908 to -893 of the rat FAS promoter is a STAT5 binding site which confers the negative transcriptional regulation of FAS by PRL. These results support our hypothesis that STAT5 directly represses expression of FAS in adipocytes.

The association of increased transcription of FAS in rodent obesity (Guichard *et al.*, 1992) and the inhibition of adipogenesis by an allosteric inhibitor of FAS (Liu *et al.*, 2004) are highly indicative that regulation of FAS expression and activity in adipocytes is an important control of energy homeostasis. The characterization of a STAT5 binding site in the rat FAS promoter identifies a novel mechanism for repression of FAS expression. The -908 to -893 site of the rat FAS promoter is also present in the murine FAS promoter (AL663090) at position -895 to -887. Interestingly, the human FAS promoter (AF250144) contains a site at -1091 to -1083 (TTCGAGGAA) which is different from the sequence identified from the rodent promoters, but complements the STAT consensus sequence, TTCNNNGAA. Hence, although the precise sequence of the STAT5 binding site is not conserved across species, the binding by STAT5 to these sites in the FAS promoter may be an evolutionarily retained mechanism of regulating FAS expression in adipose tissue.

Our observation that STAT5A, not other adipocyte STATs, preferentially binds to the -908 to -893 site (Fig. 5.5) is consistent with previous studies demonstrating that STAT proteins

bind similar sequences but exhibit subtle differences in affinity for nucleotides between and beyond the half sites of the palindrome (Soldaini *et al.*, 2000; Ehret *et al.*, 2001). This specificity in STAT binding may account for the distinct repertoire of target genes regulated by each STAT protein (Ehret *et al.*, 2001). Our data suggest that the regulation of transcription mediated by the -908 to -893 region of the rat FAS promoter is primarily regulated by STAT 5A binding.

PRL has been shown to have anti-lipogenic effects in adipose tissue through inhibition of LPL expression and the repression of FAS (Flint *et al.*, 1981) and LPL activity (Ling *et al.*, 2003). Our results strongly suggest that PRL modulates the expression of FAS in adipocytes through STAT5A and support a role for STAT5 as a regulator of energy balance in adipocytes. PRL appears to have dual functions, positively and negatively affecting adipocyte gene expression. The association of STAT5 proteins with coactivators, corepressors and other transcription factors likely affect the ability of STAT 5A to have adipogenic and anti-adipogenic effects. Recent work from our laboratory has demonstrated that the association between STAT5A and the glucocorticoid receptor (GR) is highly regulated during fat cell differentiation (Floyd *et al.*, 2003). Hence, cooperation between STAT5A and GR may occur in the modulation of FAS since glucocorticoids have been demonstrated to affect FAS transcription and activity in adipose tissue (Volpe *et al.*, 1975; Wang *et al.*, 2004). In summary, we have observed that PRL represses expression of FAS in adipocytes and negatively regulates the rat FAS promoter. Our identification of a STAT5 binding site in the promoter of FAS characterizes a novel mechanism of regulating FAS expression. We hypothesize that the regulation of FAS by STAT5 is likely an important contribution to the maintenance of energy homeostasis.

CHAPTER 6: THE EFFECTS OF LEUKEMIA INHIBITORY FACTOR ON ADIPOCYTES

6.1 Introduction

Leukemia inhibitory factor (LIF) is a member of the gp130 cytokine family, cytokines which are unrelated by sequence, but are structurally similar and share a common receptor, gp130 (Robinson *et al.*, 1994; Kishimoto *et al.*, 1995). LIF has multiple effects on adipocytes and adipose tissue, stimulating a wasting weight loss in mice engrafted with a tumor cell line that overproduces LIF (Mori *et al.*, 1989), inhibiting lipoprotein lipase (LPL) expression and activity in 3T3-L1 and 3T3-F442A adipocytes (Marshall *et al.*, 1994), promoting adipogenesis of ob1771 cells (Aubert *et al.*, 1999), and preventing adipogenesis in bone marrow stromal cells (Gimble *et al.*, 1994). These findings suggest that the diverse effects of LIF on adipocytes depend on the developmental stage of the cells or tissue.

Recent work in our laboratory has examined the effects of two other gp130 cytokines, ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1), on adipocytes (Zvonic *et al.*, 2003; Zvonic *et al.*, 2004). CNTF increased expression and activation of insulin signaling proteins in 3T3-L1 adipocytes, but decreased expression of fatty acid synthase (FAS) and sterol regulatory element binding protein-1 (SREBP1). Like CNTF, CT-1 decreased expression of FAS protein but also decreased expression of insulin receptor substrate-1 (IRS-1) in 3T3-L1 adipocytes, indicating both overlapping and divergent effects of gp130 cytokines on adipocytes. To further elucidate the role of gp130 cytokines on adipocytes, we have investigated the action of LIF on 3T3-L1 adipocytes. As previously observed with CT-1 and CNTF, LIF neither promoted nor attenuated adipogenesis, in contrast to previous findings for LIF (Gimble *et al.*, 1994; Aubert *et al.*, 1999). We have also shown that FAS and SREBP1 protein levels decreased after a chronic treatment with LIF. In addition, both suppressor of cytokine signaling-3 (SOCS3)

and CCAAT/enhancer binding protein- δ (C/EBP δ) mRNA were rapidly induced following treatment of adipocytes with LIF. We identified three signal transducer and activator of transcription-1 (STAT1) binding sites in the C/EBP δ promoter at positions -696 to -679, -810 to -793, and -1491 to -1475. Unlike other gp130 cytokines, LIF had no effect on basal or insulin-stimulated glucose uptake, or on the expression of glucose transporter 4 (GLUT4). In summary, our results demonstrate novel and specific effects of LIF on adipocytes, suggesting a role for LIF as a regulator of lipid synthesis and an effector of signal transduction in adipocytes.

6.2 Results

To determine if LIF treatment could modulate adipogenesis, 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of exogenous LIF. The cells were exposed to LIF (0.5 nM) for the entire course of differentiation with a fresh bolus of LIF added at the time of induction and every 24 hours after that. Whole cell extracts were harvested at the time points indicated in Fig. 6.1 and were analyzed by Western blotting. Adipogenesis was assessed by induction of peroxisome proliferator-activated receptor- γ (PPAR γ), FAS, and STAT5A proteins (Spiegelman *et al.*, 1993; Chawla *et al.*, 1994; Stephens *et al.*, 1996). Although LIF attenuated the induction of FAS at 48 hours and 72 hours, LIF did not affect the overall expression of these markers of adipogenesis. The expression of STAT3 does not change during adipogenesis (Stewart *et al.*, 1999) and is shown as a loading control (Fig. 6.1). Phosphorylation of STAT3 was detected in preadipocytes following 15 minutes of stimulation with LIF. A longer exposure of the film detected a low level of STAT3 phosphorylation through the course of differentiation.

Since LIF is a potent activator of STATs 1 and 3 in 3T3-L1 cells (Stephens *et al.*, 1998) we examined the action of LIF on mature adipocytes. Whole cells extracts were collected from fully differentiated 3T3-L1 adipocytes that were treated with LIF for the various times indicated

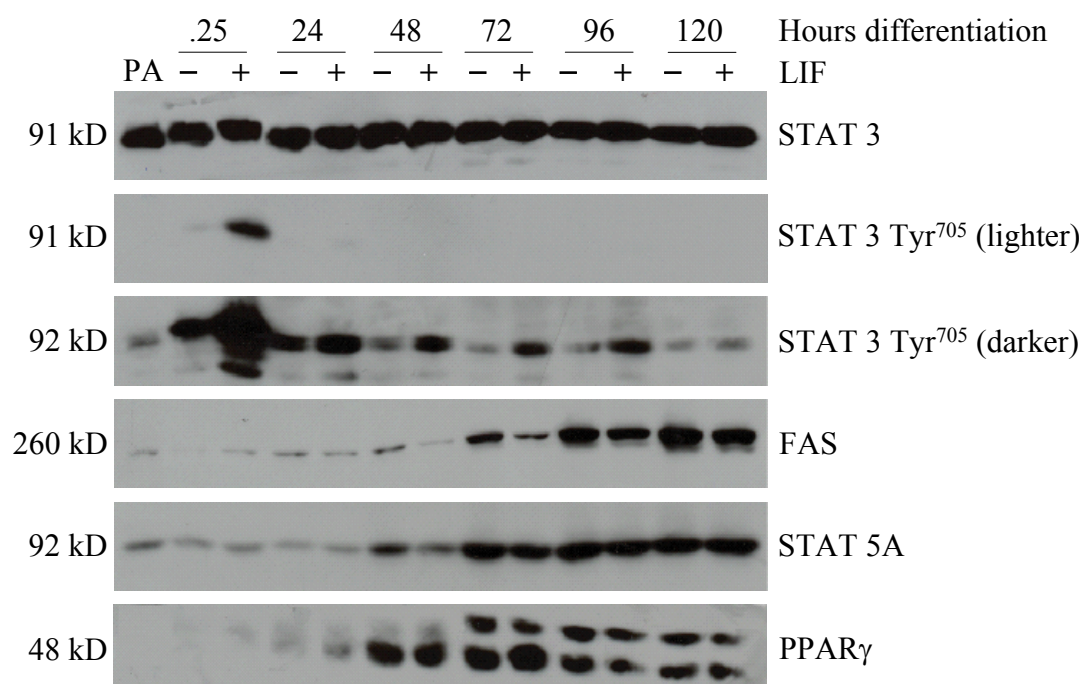


Figure 6.1 LIF does not affect the differentiation of 3T3-L1 adipocytes. Whole cell extracts were prepared from 3T3-L1 preadipocytes (PA) or 3T3-L1 cells induced to differentiate in the presence or absence of LIF (0.5 nM). Cells were treated every 24 h with a fresh bolus of LIF. Seventy-five μ g of each sample were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The detection system was horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. This is a representative experiment independently performed three times.

in Fig. 6.2. Although the phosphorylation of STATs 1 and 3 was detected at 15 and 45 minutes of stimulation, there were no changes in the level of PPAR_γ, the insulin-regulated transcription factor Foxo1, SREBP1, FAS, or acetyl coA carboxylase (ACC) proteins during the time course (Fig. 6.2). Therefore, we investigated the effect of a chronic LIF treatment on 3T3-L1 adipocytes. Fully differentiated adipocytes were stimulated with LIF for the times indicated in Fig. 6.3, with a fresh bolus of LIF added every 24 hours. Chronic LIF treatment resulted in decreased protein levels of FAS and SREBP1 at 120 hours, but the levels for Foxo1, PPAR_γ, LPL, and ACC were unchanged. Efficacy of LIF treatment is shown by the phosphorylation of STAT3 during the time course, and positive controls for activation of STATs 1, 3, and 5 are

shown with extracts from 3T3-L1 adipocytes stimulated for 15 minutes with IFN γ , LIF, and growth hormone (GH), respectively.

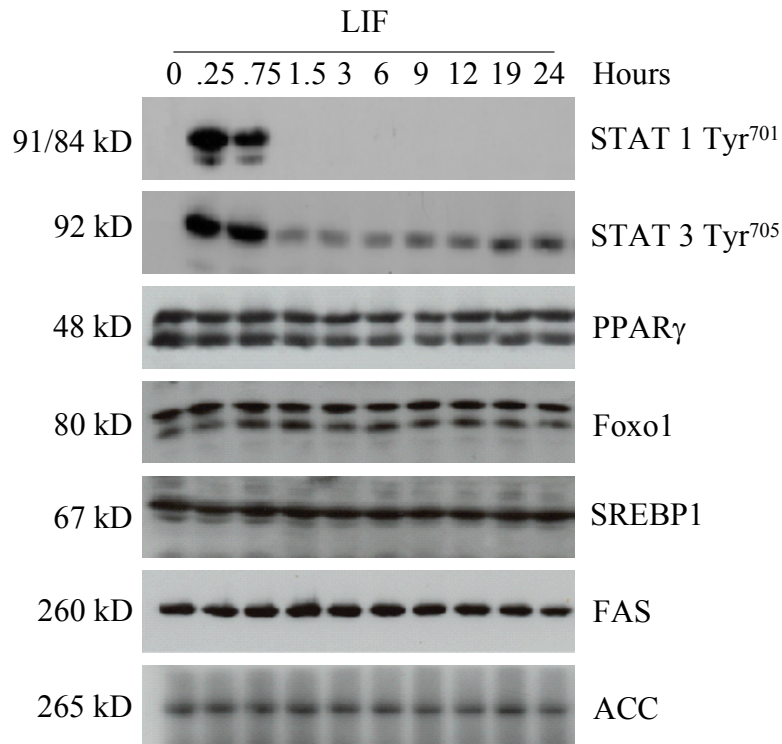


Figure 6.2 Acute LIF treatment did not affect expression of adipocyte marker proteins. Mature 3T3-L1 adipocytes were treated with LIF (0.5 nM) for the times indicated. Seventy-five μ g of protein from whole cell extracts were loaded into the gel for each sample. The samples were subjected to SDS-PAGE, and then were transferred to nitrocellulose for immunoblot analysis. This is a representative experiment independently performed two times.

Since other gp130 cytokines have been reported to have effects on insulin signaling and glucose uptake, we investigated the ability of LIF to regulate glucose uptake in 3T3-L1 adipocytes. Mature 3T3-L1 adipocytes were pretreated with LIF for 30 minutes, 48 hours or 96 hours. Adipocytes were stimulated with insulin for 15 minutes and assayed for uptake of [3 H] 2-deoxyglucose. As shown in Fig. 6.4, insulin treatment induced an approximately 4-fold increase in glucose uptake, regardless of the acute or chronic treatments of LIF (ANOVA $F(7,16) = 123.37$, $p = 1.02E-12$; Scheffé's test). We observed no significant differences for basal glucose

uptake by adipocytes. Furthermore, there were no effects on the level of GLUT4 mRNA over a time course of 24 hours (Fig. 6.5).

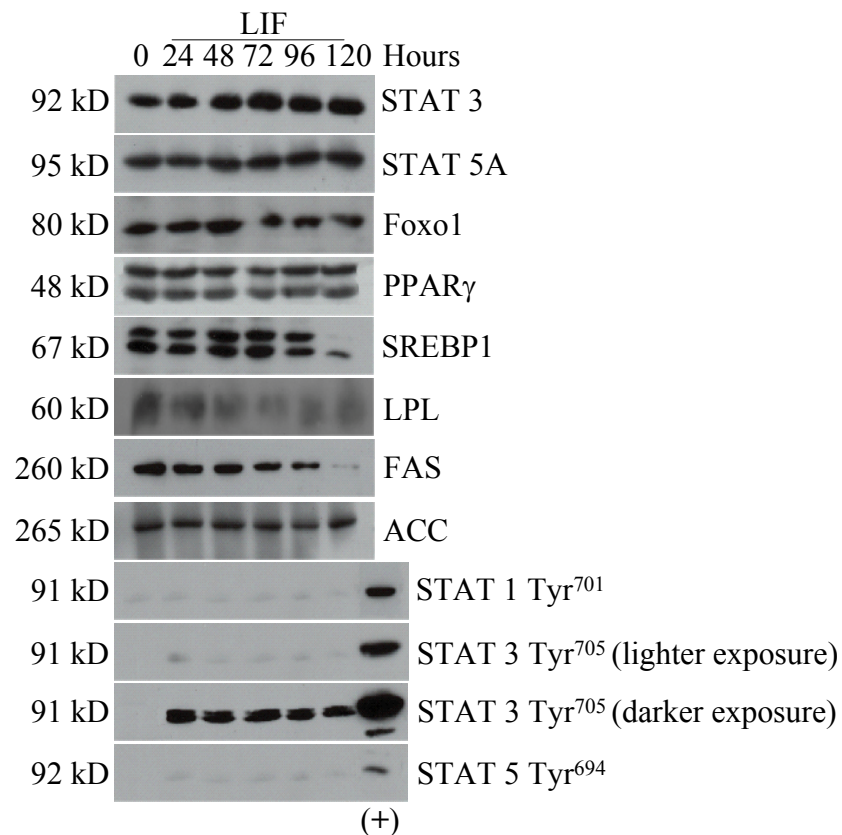


Figure 6.3 The effects of chronic treatment of LIF on 3T3-L1 adipocytes. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes following treatment with LIF (0.5 nM) for the indicated times. Cells were treated every 24 h with a fresh bolus of LIF. Seventy-five μ g of each sample were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. (+) indicates a positive control of a 15-min treatment with $\text{IFN}\gamma$, LIF, and GH for activation of STAT1, STAT3, and STAT5, respectively. This is a representative experiment independently performed two times.

To examine the effects of LIF on the regulation of mRNA for several adipocyte genes which could not be analyzed by Western blot because effective antibodies are not available for the proteins, total RNA was collected from fully differentiated 3T3-L1 adipocytes following treatment with LIF for the times indicated in Fig. 6.5. Although no changes were detected for expression of C/EBP α , C/EBP β , or the fatty acid binding protein, aP2, LIF induced a rapid and

transient upregulation of mRNA for SOCS3 and C/EBP δ within one hour of stimulation.

Furthermore, the upregulation of SOCS3 and C/EBP δ was independent of extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) activity, as pretreatment with the MAPK kinase (MEK) inhibitor, U0126, did not block the induction by LIF (data not shown). Induction of SOCS3 by gp130 cytokines has been demonstrated in adipocytes (Zvonic *et al.*, 2004) and is well characterized in other cell types (Krebs and Hilton, 2001).

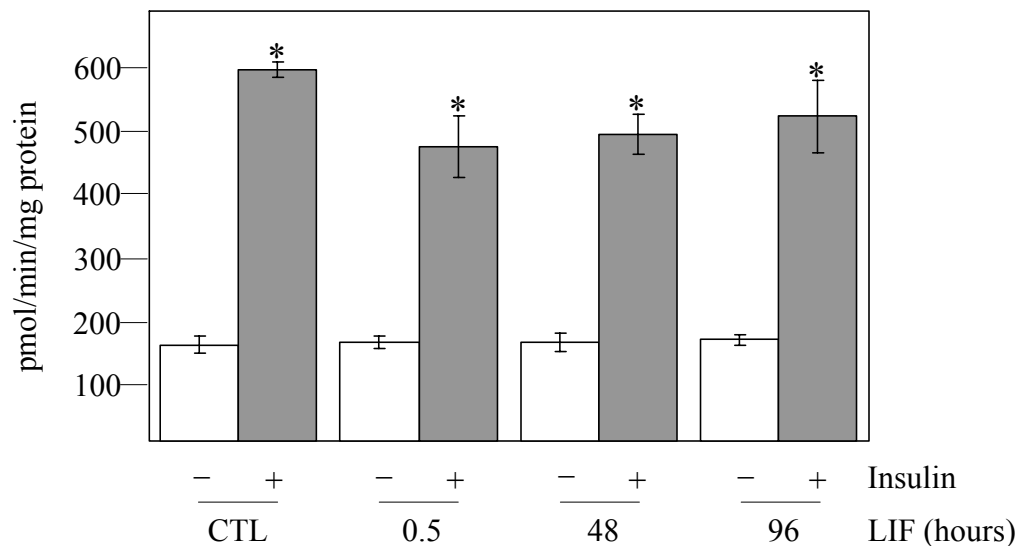


Figure 6.4 LIF does not affect insulin stimulation of glucose uptake. Fully differentiated 3T3-L1 adipocytes were treated with LIF (0.5 nM) for the times indicated. Cells were treated every 24 h with a fresh bolus of LIF. Cells were serum deprived 4 h and then stimulated with insulin (44 nM) for 15 min. Glucose uptake was initiated by addition of [3 H] 2-deoxyglucose. Glucose uptake for each time point was measured in triplicate and is given as the mean \pm the standard deviation. Asterisks indicate statistically significant differences compared to untreated samples (*, $p < 0.01$; Scheffé's test; ANOVA $F(7,16) = 123.37$, $p = 1.02E-12$). This is a representative experiment independently performed two times.

The role of STAT3 in the upregulation of C/EBP δ has been investigated in other cell types, and evidence indicated that the -115 to -98 site in the C/EBP δ promoter was bound by STAT3 following stimulation with interleukin-6 (IL-6) (Yamada *et al.*, 1997; Hutt *et al.*, 2000). Hence, we examined the ability of LIF to induce binding to the -115 to -98 element of the C/EBP δ promoter. Electrophoretic mobility shift assay (EMSA) was performed with nuclear

extracts from 3T3-L1 adipocytes treated with LIF for 15 minutes. As shown in Fig. 6.6, no detectable binding to the -115 to -98 oligonucleotide was observed with LIF treatment, indicating that this site does not contribute to the regulation of C/EBP δ by LIF in 3T3-L1 adipocytes. Thus, we looked for other possible STAT binding sites in the C/EBP δ promoter and identified four sites at positions -616 to -599, -696 to -679, -780 to -763, and -1491 to -1475 which resembled the consensus STAT recognition sequence TTCNNNGAA (Table 6.1). As shown in Fig. 6.6, LIF-induced nuclear protein complexes shifted the sites at -696 to -679, -780 to -763, and -1491 to -1475, but not the -616 to -599 oligonucleotide (data not shown). In addition, we observed faint bands of cytoplasmic proteins from LIF-treated adipocytes for the -780 to -763 and the -1491 to -1475 oligonucleotides, which may be indicative that the protein complex that binds to these sites forms in the cytoplasm.

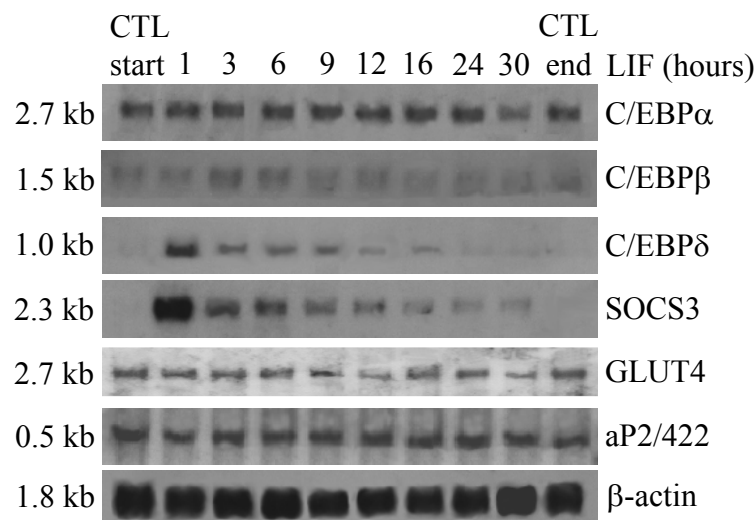


Figure 6.5 LIF induces the expression of C/EBP δ and SOCS3 mRNA. Total RNA was isolated from 3T3-L1 adipocytes following treatment with LIF (0.5 nM) for the times indicated above. Untreated cells (CTL) were harvested at the start and end of the time course. Fifteen μ g of total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis with radiolabeled probes for the indicated genes. Hybridization to β -actin is included as a loading control. This is a representative experiment independently performed two times.

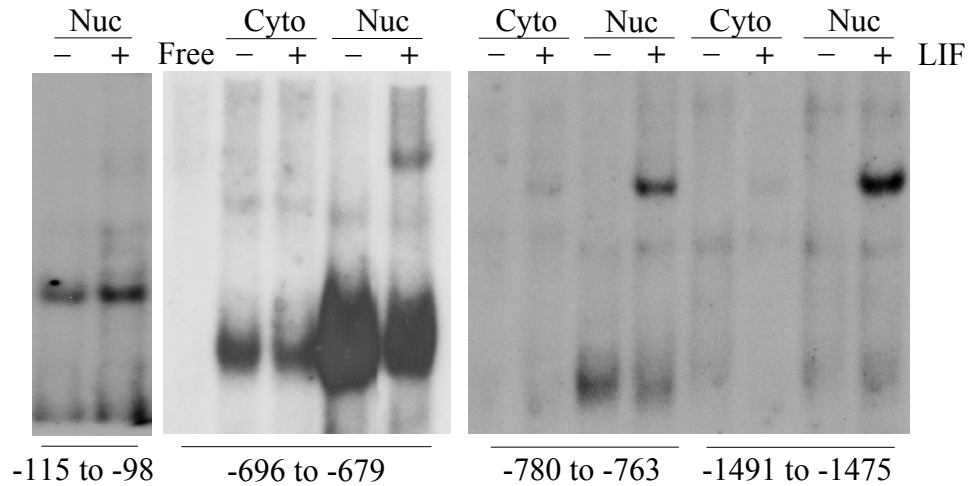


Figure 6.6 LIF activates binding by protein complexes from the nuclei of 3T3-L1 adipocytes to three sites of the C/EBP δ promoter. Nuclear extracts were prepared from differentiated 3T3-L1 adipocytes that were untreated or treated with LIF for 15 min. For each sample, 10 μ g of protein were incubated with 50,000 cpm/ml of the indicated 32 P-labeled probe of the C/EBP δ promoter. The protein-DNA complexes were resolved by electromobility shift assay. This is a representative experiment independently performed two times.

Table 6.1 STAT binding sites in the C/EBP δ promoter

Gene	Position	Sequence	LIF Responsive
Consensus	GAS/SIE	TTC NNN GAA	
C/EBP δ	-115 to -98	TCG TTC CCA GCA GCA AC	No
C/EBP δ	-616 to -599	AGC TTT AAT AAC CAA G	No
C/EBP δ	-696 to -679	AAT TTC AGA ATA ATA TCC	Yes
C/EBP δ	-780 to -763	CTT TTC ACG AAT TTT GAA	Yes
C/EBP δ	-1491 to -1475	CGT TTC CCG TAA ATC CCT CCC	Yes
α_2 -M	-168 to -148	TCC TTC TGG GAA TC	Yes
m67		CGT TTC CCG TAA ATC CCT CCC	Yes

Since LIF is a strong activator of STAT3 (Figs. 6.2 and 6.3), we hypothesized that the protein complex contained STAT3. However, supershift analysis using multiple STAT3 antibodies, along with antibodies against STAT1 and STAT5A clearly indicates that the protein complex induced by LIF primarily contains STAT1 (Fig. 6.7, panels 1-3, lane 7). As shown in Fig. 6.7, supershift was not detected with a STAT5A antibody (lane 8) or with STAT3 antibodies which recognize the carboxy terminus (lane 4), amino acids 1-175 (lane 5), or amino acids 688-722 (lane 6) of STAT3. We also examined the induction of binding by LIF-induced proteins to

two oligonucleotides that are routinely used as positive controls for STAT3 binding, the -168 to -148 site from the rat α_2 -macroglobulin (α_2 M) promoter (Hattori *et al.*, 1990) and the m67 oligonucleotide derived from the c-fos -345 to -323 promoter (Wagner *et al.*, 1990). As shown in Fig. 6.7A, LIF treatment induced binding to these oligonucleotides (panels 4 and 5, lane 3). Similar to our findings with the C/EBP δ promoter sites, only STAT1 antibody supershifted the protein complex induced by LIF for both the m67 and α_2 M oligonucleotides. To further investigate the activation of STAT3 binding by LIF, we used a longer m67 oligonucleotide for EMSA analysis (Fig. 6.7B). We detected binding by two protein complexes that were induced by LIF. The weak binding by the protein complex exhibiting slower mobility was blocked by antibodies for STAT3 (lanes 4 and 5). The more prominent protein complex exhibiting faster mobility was supershifted by STAT1 antibody (lane 6). STAT5A antibody was included as a negative control. Thus, although LIF is a potent activator of STAT3, STAT1 appears to preferentially bind under these conditions in 3T3-L1 adipocytes. Furthermore, our supershift analysis suggests that STAT1 contributes to the LIF-induced modulation of C/EBP δ in adipocytes.

6.3 Discussion

Recent work from our laboratory has demonstrated that gp130 cytokines, CT-1 and CNTF, have overlapping and divergent effects on the expression and activation of proteins of lipid and glucose metabolism in adipocytes (Zvonic *et al.*, 2003; Zvonic *et al.*, 2004). The gp130 cytokines activate the JAK/STAT pathway via the gp130 and LIFR receptors. Because LIF also signals through gp130 and LIFR, we hypothesized that LIF would regulate adipocyte proteins. Our studies with 3T3-L1 cells have shown that LIF activates STATs 1 and 3 in preadipocytes and mature adipocytes. Because LIFR is expressed more abundantly in preadipocytes (Zvonic *et*

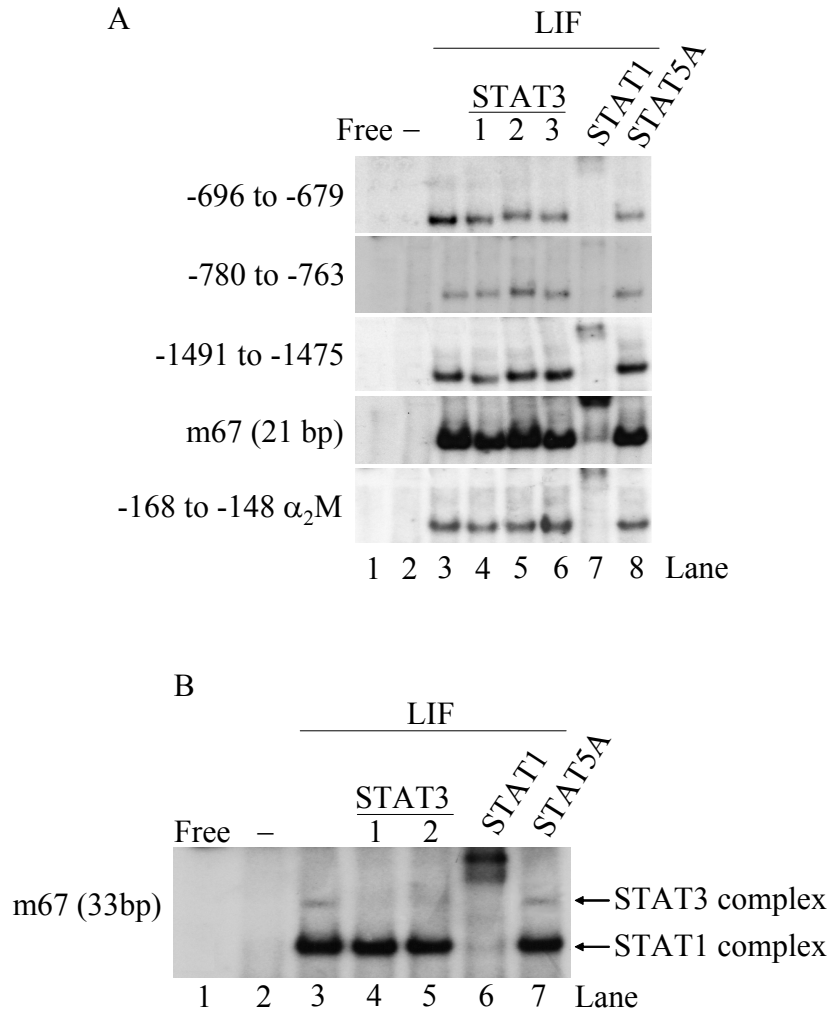


Figure 6.7 Binding of LIF-activated STAT1, not STAT3, to sites in the C/EBP δ promoter.

(A) Nuclear extracts were prepared from differentiated 3T3-L1 adipocytes that were untreated or treated with LIF for 15 min. For each sample, 10 μ g of protein were incubated with 50,000 cpm/ml of the 32 P-labeled probe (indicated above) of the C/EBP δ promoter or of the STAT3 oligonucleotides for m67 or -168 to -148 site of the rat α_2 M promoter. For supershift, samples were preincubated with the indicated antibodies (4 μ g): STAT3 1 (Santa Cruz C-20), 2 (Transduction Laboratories), 3 (Upstate); STAT1 (Upstate); and STAT5 (Santa Cruz L-20). The protein-DNA complexes were resolved by electromobility shift assay. This is a representative experiment independently performed two times. (B) Nuclear extracts from untreated or LIF-treated 3T3-L1 adipocytes were incubated with the 32 P-labeled 33-base pair m67 oligonucleotide. For supershift analysis, samples were preincubated with the indicated antibodies.

al., 2003), we predicted that LIF would regulate adipogenesis. However, there were no effects of LIF on the *in vitro* differentiation of 3T3-L1 adipocytes. In another cell line, Ob1771, LIF

promoted differentiation (Aubert *et al.*, 1999). Thus, it is possible that this discrepancy is due to differences in commitment to the adipocyte lineage of these two cell lines.

Since adipocytes are responsive to LIF, we studied the effects of this cytokine on fully differentiated adipocytes. Acute treatment did not regulate the expression of several adipocyte proteins, but expression of SOCS3 and C/EBP δ mRNA was strongly and transiently stimulated by LIF. SOCS3 is a member of the SOCS protein family which is characterized by their ability to negatively modulate cytokine signaling (Krebs and Hilton, 2001). The induction of SOCS3 expression has been demonstrated by many cytokines, and direct regulation by STAT proteins has been described (Auernhammer *et al.*, 1999). These data are consistent with recent findings from our laboratory that CNTF and CT-1 upregulate SOCS3 mRNA (Zvonic *et al.*, 2004). Interestingly, there is recent evidence that SOCS3 is a regulator of insulin signaling and may cause insulin resistance in adipocytes through its effects on insulin receptor subunit (IRS) protein expression (Shi *et al.*, 2004). Although we did not observe effects on insulin-stimulated glucose uptake by LIF, the upregulation of SOCS3 by gp130 cytokines is clearly a major component of JAK/STAT signaling in adipocytes.

C/EBP δ is a basic-leucine zipper transcription factor with a well-established role in adipogenesis (Darlington *et al.*, 1998), although knock-out studies indicate that C/EBP δ alone is weakly adipogenic (Tanaka *et al.*, 1997). The IL-6-induced upregulation of C/EBP δ in mammary epithelium (Hutt *et al.*, 2000) and HepG2 cells (Yamada *et al.*, 1997) has been shown to be mediated by the -115 to -98 region of the promoter, but our data indicated that this site was not sensitive to LIF regulation in adipocytes (Fig. 6.6). However, three other sites at positions -696 to -679, -780 to -763, and -1491 to -1475 were bound by LIF-activated protein complexes which contained STAT1 (Fig. 6.6). Interestingly, the protein complex did not contain STAT3, as

we had predicted, as LIF is a more potent activator of STAT3 than STAT1, as shown in Fig. 6.2. Moreover, we did not observe STAT3 binding to two oligonucleotides routinely used as positive controls for STAT3 binding, -168 to -148 site of the rat α_2 M promoter (Hattori *et al.*, 1990) and m67 (Wagner *et al.*, 1990). Yet, our supershift analysis revealed that these oligonucleotides were shifted by protein complexes which contained STAT1. Only when we used a long form of the m67 oligonucleotide did we detect a faint band that represented STAT3 binding. Our observations strongly suggest that STAT1 preferentially binds under these conditions in 3T3-L1 adipocytes. These results have led us to hypothesize that the three sites in the C/EBP δ promoter confer the LIF response and that binding by STAT1 may mediate the upregulation of C/EBP δ in adipocytes.

The consequences of increased expression of C/EBP δ in mature adipocytes are not known. It has been shown that tumor necrosis factor- α (TNF α) (Kurebayashi *et al.*, 2001), dexamethasone (MacDougald *et al.*, 1994), and insulin (MacDougald *et al.*, 1995) also upregulate C/EBP δ in mature adipocytes in a rapid and transient manner. Interestingly, the lack of effect of LIF on C/EBP α expression suggests that although C/EBP α is regulated by C/EBP δ in differentiating adipocytes (Lane *et al.*, 1999), it is not affected by C/EBP δ expression in mature adipocytes. In other cell types, C/EBP δ negatively regulates the expression of the α_2 chain of type I collagen, a critical structural component of the extracellular matrix (Greenwel *et al.*, 2000). C/EBP δ upregulates expression of IL-6 in astrocytes (Schwaninger *et al.*, 2000), intestinal epithelial cells (Hungness *et al.*, 2002), and osteoblasts (Ruddy *et al.*, 2004); thus, IL-6, an adipokine associated with obesity (Bastard *et al.*, 2000), may be a transcriptional target of C/EBP δ in adipocytes as well. Effects on C/EBP δ targets would likely be transient, since the induction of C/EBP δ is short-lived (MacDougald *et al.*, 1994). These potential outcomes of

increased C/EBP δ expression give insight into one means by which LIF exerts effects on adipocyte function.

We also examined the effect of a chronic administration of LIF in fully differentiated adipocytes. Both FAS and SREBP1 proteins exhibited decreased expression after 120 hours of LIF stimulation. FAS is the enzyme catalyzing all of the steps in the synthesis of palmitate from acetyl CoA and malonyl, and the level of its expression is coupled to the rate of lipogenesis (Sul and Wang, 1998). SREBP1 is a transcription factor known to modulate genes associated with fat and cholesterol metabolism (Horton *et al.*, 2002), including FAS (Bennett *et al.*, 1996). The decreased expression of SREBP1 and FAS may result in changes in lipid accumulation in adipocytes as a result of prolonged exposure to LIF *in vivo*. Since the effect of LIF on the expression of SREBP1 and FAS was not evident until 120 hours, it is unlikely that the down-regulation was mediated by STAT proteins.

In light of recent studies demonstrating upregulation of GLUT4 expression by CNTF (Zvonic *et al.*, 2003) and improved insulin-stimulated glucose uptake by CT-1 (Zvonic *et al.*, 2004), we investigated the ability of LIF to modulate glucose uptake in adipocytes. Our findings clearly demonstrate that LIF does not affect basal or insulin-stimulated glucose uptake, and does not modulate expression of GLUT4. Thus, LIF likely does not affect glucose disposal in adipose tissue.

In summary, we have demonstrated that LIF activates the JAK/STAT pathway in 3T3-L1 preadipocytes and adipocytes. Although LIF had no effect on adipogenesis of 3T3-L1 cells, LIF increased C/EBP δ expression after an acute exposure. We have identified three LIF-responsive sites in the C/EBP δ promoter that are distinct from the previously reported STAT3 binding site for IL-6 regulation in mammary epithelial cells (Hutt *et al.*, 2000) and hepatocytes (Yamada *et*

al., 1997). Moreover, these three newly identified and LIF-responsive sites bind STAT1, rather than STAT3. LIF, similar to CT-1 and CNTF, regulated proteins involved in lipid accumulation, SREBP1 and FAS, which may be indicative of a redundant role of gp130 cytokines in the modulation of adipocyte function. However, unlike these cytokines, LIF had no effect on basal or insulin-stimulated glucose uptake. These results were somewhat unexpected, given that all three cytokines signal through the same receptor components. Yet each cytokine activates a unique pattern of STATs in adipocytes. CNTF only activates STAT3 (Zvonic *et al.*, 2003), whereas LIF activates STATs 1 and 3, and CT-1 activates STATs 1, 3, and 5 (Zvonic *et al.*, 2004). Therefore, we predict that the variation in recruitment of these transcription factors mediates the nonredundant, downstream effects of gp130 cytokines in 3T3-L1 adipocytes. Furthermore, crosstalk with other signaling pathways may contribute to the divergent effects of gp130 cytokines. Current studies are underway to investigate the crosstalk among gp130 cytokines in adipocytes.

CHAPTER 7: SUMMARY AND CONCLUSIONS

Recent advances in our understanding of adipocyte biology have brought closer the possibilities of treating obesity, but have also emphasized the need for further research to better understand the complexities of mature adipocytes. Adipose tissue is highly dynamic, responding to and directing changes in energy metabolism. These responses culminate from changes in gene expression regulated by transcription factors. Thus, the activation and action of transcription factors in adipocytes is an important focus of research. The results presented in this dissertation describe STAT binding sites in the promoters of four adipocyte genes.

Five members of the STAT transcription factor family are known to be expressed in adipocytes; STATs 1, 5A, and 5B are highly induced during adipogenesis, and STATs 3 and 6 are expressed in preadipocytes and adipocytes (Stephens *et al.*, 1996; Stewart *et al.*, 1999). The expression of these proteins is strongly indicative of a role for STATs in modulating genes in adipocytes. STAT5A has been demonstrated to induce adipogenesis (Floyd and Stephens, 2003). Yet, specific STAT 5 targets have not been identified. Moreover, the function of STATs in mature adipocytes is not known. Our characterization of STAT binding sites in the promoters of PPAR γ 2, LPL, FAS and C/EBP δ strongly suggest that these adipocyte genes are regulated by STATs *in vivo*.

The negative regulation of PPAR γ , LPL and FAS by cytokines implicates STATs as repressors of transcription. Although STATs are best known as activators of transcription, negatively regulated gene targets have been identified in other tissues (Sharma *et al.*, 1998; Ikeda *et al.*, 1999; Ramana *et al.*, 2000). It is speculated that STATs repress transcription by interacting with corepressors, such as Blimp-1, myc-binding protein-1 (MBP-1), silencing mediator for retinoid and thyroid hormone receptors (SMRT), protein inhibitor of activated

STAT-y (PIASy) (Ramana *et al.*, 2000; Dong and Tweardy, 2002; Liu *et al.*, 2001) or that naturally occurring truncations lacking the transcriptional activation domain bind promoter elements to inhibit transcription (Kisseleva *et al.*, 2002). We hypothesize that in adipocytes, negative regulation by STATs occurs through the recruitment of corepressors since we have not detected the expression of truncated STAT isoforms. The identification of proteins which bind STATs in the nucleus would further elucidate the mechanism of STAT-regulated transcription.

We have demonstrated that PPAR γ is a negatively regulated target of STAT1 in 3T3-L1 adipocytes through the identification of a STAT1 binding site at the -221 to -207 position of the PPAR γ 2 promoter. This site was bound by nuclear STAT1 activated by IFN γ , CT-1, and LIF. However, binding to this site correlated with transcriptional repression by IFN γ and CT-1 but not LIF, which had no detectable effects on PPAR γ expression. Our studies have shown that the PPAR γ 2 promoter contains a STAT1 binding site that likely mediates transcriptional repression of PPAR γ by CT-1 and IFN γ . Although LIF-activated STAT1 bound to the site in the PPAR γ 2 promoter, LIF is a much less potent activator of STAT1 than IFN γ (Hogan and Stephens, 2001). Hence, an insufficient level of STAT1 may be present in the nucleus following stimulation with LIF in order to regulate PPAR γ expression. Since decreased cellular levels of PPAR γ confer resistance to weight gain (Miles *et al.*, 2000; Kubota *et al.*, 1999), we speculate that STAT1 serves to prevent expansion of adipose tissue, in part through the downregulation of the adipogenic transcription factor, PPAR γ .

Multiple studies have demonstrated a decrease in LPL mRNA expression and activity following stimulation of adipocytes with IFN γ . We have demonstrated a decrease in LPL protein following IFN γ treatment of 3T3-L1 adipocytes. Furthermore, we have identified a STAT1 binding site in the LPL promoter at position -938 to -921. LPL is the rate-limiting enzyme

catalyzing the hydrolysis of serum triglycerides for uptake by underlying tissues. Therefore, the downregulation of LPL by STAT1 would ostensibly decrease uptake of fatty acids for storage in adipose tissue. Furthermore, we propose that STAT1 mediates the lipolytic effects of IFN γ in fat cells and may function to limit storage of lipid in adipose tissue.

We have demonstrated that FAS expression decreases following treatment of 3T3-L1 adipocytes with GH and PRL, potent activators of STAT5 proteins. Our studies with serial deletions of the FAS promoter reporter constructs indicated the presence of a PRL responsive region between -1594 and -700 basepairs of the FAS promoter. We identified a STAT5 binding site at position -908 to -893, which was bound by a protein complex containing STAT5A. Moreover, the functional significance of this site was determined by the site-directed mutation of two basepairs within the -908 to -893 site in a FAS promoter reporter construct. FAS is the central enzyme of *de novo* lipogenesis, and although the majority of lipid stored in adipose tissue comes from diet, *de novo* lipogenesis contributes to the accumulation of stored lipid (Wang *et al.*, 2004). Previous studies have clearly identified STAT5A as an adipogenic transcription factor, but our results indicate that STAT5A may also be anti-lipogenic in mature adipocytes.

Finally, we examined the effects of LIF, a gp130 cytokine, on 3T3-L1 adipocytes. LIF robustly activates STAT3, but also activates STAT1. We did not observe significant effects of LIF on adipogenesis of 3T3-L1 adipocytes. Furthermore, LIF did not acutely affect the expression of several adipocyte marker proteins and did not modulate basal or insulin-stimulated glucose uptake. However, following a chronic stimulation with LIF, the expression of SREBP1 and FAS were notably decreased. In addition, we observed the rapid and transient upregulation of SOCS3 and C/EBP δ mRNA. Although a previously described STAT3 site in the C/EBP δ promoter did not bind LIF-activated protein complexes, three new sites in the promoter at

positions -696 to -679, -810 to -793, and -1491 to -1475 were bound by LIF-activated STAT1 from 3T3-L1 adipocytes. Thus, we propose that these sites confer the positive regulation of C/EBP δ by LIF. It is unclear what the consequence is of transient C/EBP δ upregulation in adipocytes, but it has been shown to also occur following stimulation of adipocytes with TNF α , insulin, and dexamethasone and may result in remodeling of the extracellular matrix, changes in stability of chromatin, or altered levels of cytokine production.

The induction of STATs 1 and 5 during adipogenesis strongly suggested that these STATs would contribute to adipocyte biology. The identification of adipocyte genes as targets of regulation by STATs clearly implicates STATs 1 and 5A as important modulators of adipocyte biology. Although STATs 3 and 6 are also expressed in adipocytes, the expression of these transcription factors is not regulated during adipogenesis. Activation of STAT6 in mature adipocytes has not been reported, and we have not identified STAT3-regulated genes. Hence, it is possible that STATs 3 and 6 may have functions in preadipocytes or serve to maintain general cell functions, instead of modulating specific adipocyte genes. Identification of other gene targets of STATs in adipocytes may reveal other roles for STATs. The epidemic of obesity underscores the importance of continued research in all areas of adipocyte biology, including effects of cytokines and the role of STATs in adipocytes.

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VITA

Jessica Hogan received her Bachelor of Science degree with a major in psychology and a minor in cellular and molecular biology from Southeast Missouri State University in 1997. She continued her education at Middle Tennessee State University, joining the laboratory of Dr. William Stewart. In 2000, she obtained a Master of Science degree in molecular and cellular biology for her study of signal transducers and activators of transcription (STATs) in Schwann cells. Jessica then joined the laboratory of Dr. Jacqueline Stephens in the Department of Biological Sciences at Louisiana State University as a doctoral student. The focus of her dissertation research was the identification of STAT target genes in adipocytes. Jessica will complete her studies and earn the degree of Doctor of Philosophy in biochemistry in May of 2005. In January of 2005, she plans to work for Dr. Leslie Kozak at Pennington Biomedical Research Center.