Acylation Stimulating Protein: Identification of a Novel Effect, Receptor Interaction and Intracellular Signaling

by

Magdalena H. Maslowska

Department of Medicine, Division of Experimental Medicine

McGill University, Montreal, Quebec

August 2003

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Your file Votre référence
ISBN: 0-612-98322-6
Our file Notre référence
ISBN: 0-612-98322-6

"It is through science that we prove,

but through intuition that we discover"

Henri Poincare
mathematician, 1854-1912

DEDICATION

To my children, Natasha and Jonathan

ABSTRACT

Obesity is an ever-growing problem of our society and, therefore, understanding the underlying endocrine causes is vital to both the prevention and treatment of this disease. This present thesis work was undertaken to better understand the impact of Acylation Stimulating Protein (ASP), an adipokine, on the physiology and pathophysiology of the adipose tissue. I have demonstrated, for the first time, that ASP participates in the recruitment of preadipocytes to become adipocytes (differentiation process). Although, the ASP effects were comparable to those of insulin, preliminary microarray analysis indicates that the early intracellular signaling pathways are likely quite different for both hormones. Furthermore, I have identified and begun to characterize an ASP receptor initially identified as C5L2. I have demonstrated that binding affinity of ASP and C3a (immediate ASP precursor) to C5L2 is similar to that observed in cells that are responsive to ASP (triglyceride synthesis stimulation, TGS). I have also shown, for the first time, that C5L2 is expressed in human adipose tissue, human skin fibroblasts and 3T3-L1 preadipocytes. Finally, the ASP signaling pathway resulting in increased TG synthesis has began to be explored using a "shot gun" approach. This initial phase of research has provided evidence of the involvement of phospholipase C and phosphoinositide 3-kinase in ASP signaling. Overall, the data presented in this thesis defines more clearly ASP-cell interaction and reveals potential targets for pharmacological agents that may prevent or slow down development of obesity in the future.

RESUME

L'obésité est un problème sans cesse croissant dans nos sociétés modernes. Aussi, la compréhension des causes endocrines sous-jacentes s'avère essentielle à la prévention et au traitement de la maladie. Le travail présenté dans cette thèse a été entrepris pour mieux définir l'impact de l'adipocine ASP (*acylation stimulating protein*) sur la physiologie et la pathophysiologie du tissu adipeux. Nous démontrons pour la première fois que l'ASP participe au recrutement des préadipocytes pour leur processus de différenciation en adipocytes. Bien que les effets de l'ASP soient comparables à ceux de l'insuline, des analyses préliminaires par *microarray* indiquent que les voies intracellulaires de signalisation précoce semblent tout à fait différentes pour les deux hormones. De plus, nous avons identifié et débuté la caractérisation d'un récepteur à l'ASP, initialement nommé C5L2. Nous démontrons que l'affinité de liaison de l'ASP et du C3a (un précurseur immédiat de l'ASP) au récepteur C5L2 apparaît similaire aux observations dans les cellules sensibles à l'ASP (qui stimulent la synthèse de triglycérides). Nous démontrons également pour la première fois que le C5L2 est exprimé dans le tissu adipeux et le fibroblaste humain de même que dans les préadipocytes 3T3-L1. Enfin, l'exploration de la voie de signalisation de l'ASP qui conduit à une augmentation de la synthèse de triglycérides a été amorcée par l'approche *shot gun*. Cette phase initiale de recherche a fourni des évidences sur l'implication de la phospholipase C et de la 3-phosphoinositide kinase dans les mécanismes de signalisation cellulaire de l'ASP. Globalement, les résultats présentés dans cette

thèse définissent plus clairement les interactions ASP-cellules et révèlent ainsi des cibles potentielles pour des agents pharmacologiques visant à prévenir ou ralentir le développement de l'obésité.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3T3-L1	differentiation-competent murine cell line
3T3-F442A	differentiation-competent murine cell line
ASP	Acylation stimulating protein
C3	Complement factor C3
C3a	Fragment of the complement factor C3
C3a desArg	Desarginated form of C3a
C3aR	C3a receptor
C5L2	Novel chemokine binding orphan receptor
cAMP	Adenosine 3', 5' – cyclic monophosphate
C/EBP	CCAAT enhancer binding protein
DAG	Diacylglycerol
DGAT	Diacylglycerol acyltransferase
DX	Dexamethasone
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acid
G-3-P	Glycerol 3-phosphate
GDP	Guanosine diphosphate
GPAT	Glycerol 3-phosphate acyltransferase
Glut	Glucose transporter
GTP	Guanosine triphosphate
HEK 293	Human kidney cell line
HSF	Human skin fibroblasts

HSL	Hormone sensitive lipase
IBMX	Isobutylmethylxanthine
IC_{50}	Inhibition constant at which 50% of receptor is in a bound form
K_d	Binding affinity constant
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
IP	Inositol phosphate
MAPK	Mitogen-activated protein kinase
PA	Phosphatidic acid
PDK-1	3-phosphoinositide dependent protein kinase-1
PI3K	Phosphoinositide 3-kinase
PIP	Phosphatidylinositol phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PL	Phospholipase
PMA	Phorbol 13-myristate 12-acetate
PPAR	Peroxisome proliferator activated receptor
PT	Pertussis toxin
TG	Triglyceride
TGS	Triglyceride synthesis
TNF-α	Tumor necrosis factor α

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Katherine Cianflone for being an amazing supervisor. It is from her, that over the years I had a rare opportunity to develop an appreciation for the simplicity of the complex scientific concepts and the critical thinking necessary to understand them. She was always present, full of encouragement, dedicating endless time to discussions and bouncing off ideas, never losing the focus and providing a never-ending enthusiasm in rough times.

I would also like to acknowledge Dr. Allan Sniderman contribution to my scientific development. It is from many of his ideas and concepts that I have developed a better understanding of the physiology and pathophysiology of the systems we were studying. I would like to thank him for rounding the big picture and providing "from bench to bed" interpretations.

Special thanks to David Kalant for making sure we had all the supplies and working equipment ready, for iodinating ASP and developing ASP binding assay. Steve Philis and Robert Zakarian contributed by providing the research team with purified and active ASP. Farzad Assadi and Robert Finkelstein helped in processing experiments for triglyceride synthesis assay. May Faraj, Carrie Matheson and Thea Scantlebury offered their friendship in good times and in bad. Gina Ciavarella graciously helped with generating references.

Finally, I would like to thank many of my colleagues that I had a pleasure working with at the Mike Rosenbloom laboratory for cardiovascular research.

Preface I

This thesis was prepared in a manuscript-style form

CONTRIBUTION OF AUTHORS

The following manuscripts are presented as a part of this thesis in chapters 2, 3 and 4. Contribution of each of the authors is acknowledged:

Chapter 2: *Novel role for Acylation Stimulating Protein: an in vitro regulator of adipocyte differentiation.* Maslowska M, Wang HW, Sladek R, Cianflone K. Endocrinology 2003 (revisions requested).

The ever-growing experimental evidence of the importance of the ASP as a regulator of lipid metabolism has been instrumental in shaping our views on ASP action. I, Magdalena Maslowska, have proposed the hypothesis that ASP is the *in vivo* physiological trigger of adipocyte differentiation and performed pilot experiments showing evidence that ASP can induce differentiation of primary human preadipocytes into adipocytes in an *in vitro* situation. Subsequently, my role in this project was to select an appropriate cell culture model, design, plan and supervise the execution of all the experiments necessary to prove our hypothesis. Not only have I ensured proper data collection but I have also been responsible for data interpretation, statistical analysis, graphic representation of the results, literature review and manuscript preparation. Hong Wai Wang had performed majority of planned experiments and collected the data, Rob Sladek

performed microarray analysis and Dr Katherine Cianflone supervised the entire project and helped with manuscript preparation.

Chapter 3: *The Chemoattractant Receptor-like Protein, C5L2 Binds C3a des-Arg^{77}/ Acylation Stimulating Protein.* Kalant D*, Cain S*, Maslowska M*, Cianflone K, Monk P. J Biol Chem 278 (13): 11123-9, 2003. (* authors contributed equally to this work).

Finding the identity of the ASP receptor has been a focus of an intensive research of this laboratory over the course of more than 4 years. Although we have taken various approaches (biochemical and molecular biology) to identify the ASP receptor, a collaboration with Dr P. Monk who was studying a novel orphan receptor C5L2, led to the identification of an ASP receptor. Findings presented in this paper are the result of intensive work of a number of individuals and will only be the first in a series demonstrating C5L2 role in ASP action. More specifically:

David Kalant performed binding experiments with FLUOS-labeled ASP and C3a and C5L2 cell surface detection with FLUOS-labeled C5L2 antibody in HEK293, HEK293–C5L2 and human skin fibroblasts. In addition, he analyzed C5L2 expression level. Stuart Cain performed competition binding experiments with radiolabelled C3a, ASP (C3a desArg) and related ligands in RBL-2H3 cells transfected with C5L2, C3a receptor and CD88 (C5a receptor). He was also involved in performing the cellular activation assays ((-hexoaminidase release). I, Magdalena Maslowska, have carried out all of the competition binding

experiments with radiolabelled ASP (C3a desArg) and C3a in various cell systems (human skin fibroblasts, U937, Bt_2cAMP-U937, HEK293, HEK293 stably transfected with C3a receptor). I have also done triglyceride synthesis (TGS) assays for C3a and ASP (C3a desArg) in the same cell types as in competition binding assays and performed TGS assays for C3a, ASP (C3a desArg), C4a, C4a desArg, C5a, C5a desArg in 3T3-L1 preadipocytes.

More importantly, each of the authors was responsible for the design of experiments, troubleshooting, cell culture, experimental execution, data collection and interpretation, statistical analysis (Magdalena Maslowska only), literature review and write-up (methods, results and discussion) pertinent to work performed by each person. Finally, Dr. Katherine Cianflone and Dr. Peter Monk provided supervision and were instrumental in putting all the experimental data in a cohesive and comprehensible manner as a completed manuscript.

Chapter 4: *Targeting the ASP triglyceride signaling pathway.*

Work in progress 2003.

In this project, together with my supervisor Dr. Katherine Cianflone, I have outlined possible pathways, selected specific inhibitors, designed, planned and performed majority of the experiments, collected, analyzed and interpreted the data, and prepared figures and manuscript for publication. Farzad Assadi, who completed a 6-month training period in the laboratory, was responsible for processing some of the TG synthesis assays and also contributed intellectually.

PUBLICATIONS

The following is a summary of all the articles and abstracts, which I have co-authored over the course my Ph.D. studies:

Articles

1. Maslowska M, Wang HW, Sladek R, Cianflone K: Novel role for Acylation Stimulating Protein: an *in vitro* regulator of adipocyte differentiation. Metabolism 2003 (submmited).
2. Kalant D*, Cain AS*, Maslowska M*, Cianflone K, Monk P: The chemoattractant receptor-like protein, C5L2 binds C3a des-Arg^{77} / Acylation stimulating protein. (* authors contributed equally to this work) J Biol Chem 278(13): 11123-9, 2003.
3. Kalant D, Maslowska M, Scantlebury T, Wang HW, Cianflone K: Control of lipogenesis in adipose tissue and the role of acylation stimulating protein. Canadian J Diabetes 27(2):154-171, 2003.
4. Sniderman AD, Maslowska M, Cianflone K: Of mice and men (and women) and the acylation-stimulating protein pathway. Curr Opin Lipid. 11: 291-296, 2000.
5. Maslowska M, Cianflone K: Acylation stimulating protein (ASP): Role in adipose tissue. Book chapter in Progress in Obesity Research: 8, (eds.) Guy-Grand B and Ailhaud G. 1999, John Libbey & Co. Ltd. / 8th Int Congress on Obesity, 65-70.
6. Maslowska M, Vu H, Phelis S, Sniderman AD, Rhode BM, Blank D, Cianflone K: Plasma acylation stimulating protein, adipsin and lipids in non-obese and obese populations. Eur J Clin Invest. 29(8): 679-86, 1999.

7. Cianflone K, Maslowska M, Sniderman AD: Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. Semin Cell Dev Biol. 10(1): 31-41, 1999.

Abstracts

1. 3rd Annual McGill Biomedical Graduate Conference, McGill University, Montréal, Québec, Canada. Novel role for Acylation Stimulating Protein: an in vitro paracrine/autocrine regulator of adipocyte differentiation. February 2003.
2. Annual Fat Club meeting, University of Montreal, St. Hyacinth, Quebec, Canada. ASP stimulates differentiation in 3T3-L1 and 3T3-F442A cells. May 2002.
3. NAASO Annual Meeting, Long Beach, California, USA. Evidence for a Unique Acylation Stimulating Protein Receptor. October/November 2000.

Chapter 1

INTRODUCTION

AND

LITERATURE REVIEW

Adipose Tissue Role in Energy Metabolism

1.1 Adipose tissue as an energy storage depot

Adipose tissue plays a major role in regulating the energy metabolism primarily by having the capacity to store excess energy as triglycerides (TG) during times of nutritional abundance, and to release the fatty acids to supply body with energy (through oxidation) for numerous vital cellular processes. The storage capacity of adipose tissue is enormous due to the involvement of the cells comprising it, namely adipocytes and preadipocytes. On one hand, hormonally regulated processes such as lipogenesis (fatty acid storage) and lipolysis (fatty acid release) take place in mature fat cells (adipocytes). In a postprandial state free fatty acids and glucose provide the building blocks for fatty acid storage (lipogenesis) and in fasting periods hydrolysis of triglycerides liberates energy in the form of fatty acids (lipolysis) for muscle, heart and other tissues. On the other hand, another event also happens, the recruitment of precursor cells, preadipocytes, to become adipocytes, which provides the adipose tissue with added storage potential.

1.1.1 Postprandial fatty acid metabolism

Following intake of a meal (postprandially), dietary fat is packaged in the intestine into lipoprotein particles, called chylomicrons, to be subsequently released into the lymph and carried into the general circulation (1). A chylomicron particle is made up of a hydrophobic core containing mainly triglyceride

molecules surrounded by a hydrophilic surface composed of phospholipids, cholesterol and a number of apolipoproteins, which in turn are necessary for interaction with various cell surface receptors or enzymes (2). Once in the circulation, chylomicron triglycerides are rapidly hydrolyzed and free fatty acids (FFA) are liberated in the capillaries by the action of lipoprotein lipase (LPL) enzyme (3). Although majority of LPL is synthesized by adipose tissue and muscle, it is secreted by the cells and subsequently translocated onto the lumenal surface of the endothelial cells lining capillaries where it resides bound to a specific glucosaminoglycan, heparan sulfate (4-6). The levels and/or activity of LPL in the adipose tissue capillaries are very high postprandially mainly due to the action of insulin (7). As a result of TG hydrolysis by LPL, FFA are liberated to be either taken up by the adipocytes for storage, or join the pool of plasma FFA and circulate bound to albumin to be eventually taken up by other cells and used for their energy requirements. Excessive influx of fatty acids into the liver will give rise to another lipoprotein particle - VLDL (very light density lipoprotein), which are then secreted by the liver into the circulation (8).

1.1.2 Lipogenesis

Intracellular stores of TG reside in specialized lipid droplets surrounded by a phospholipid monolayer with various imbedded proteins such as perilipin (9). The TG molecule itself is made up of 3 molecules of FFA esterified onto a glycerol-3-phosphate (G-3-P) backbone through the coordinated action of various enzymes (Figure 1.1). FFA used for TGS in esterification reactions are either

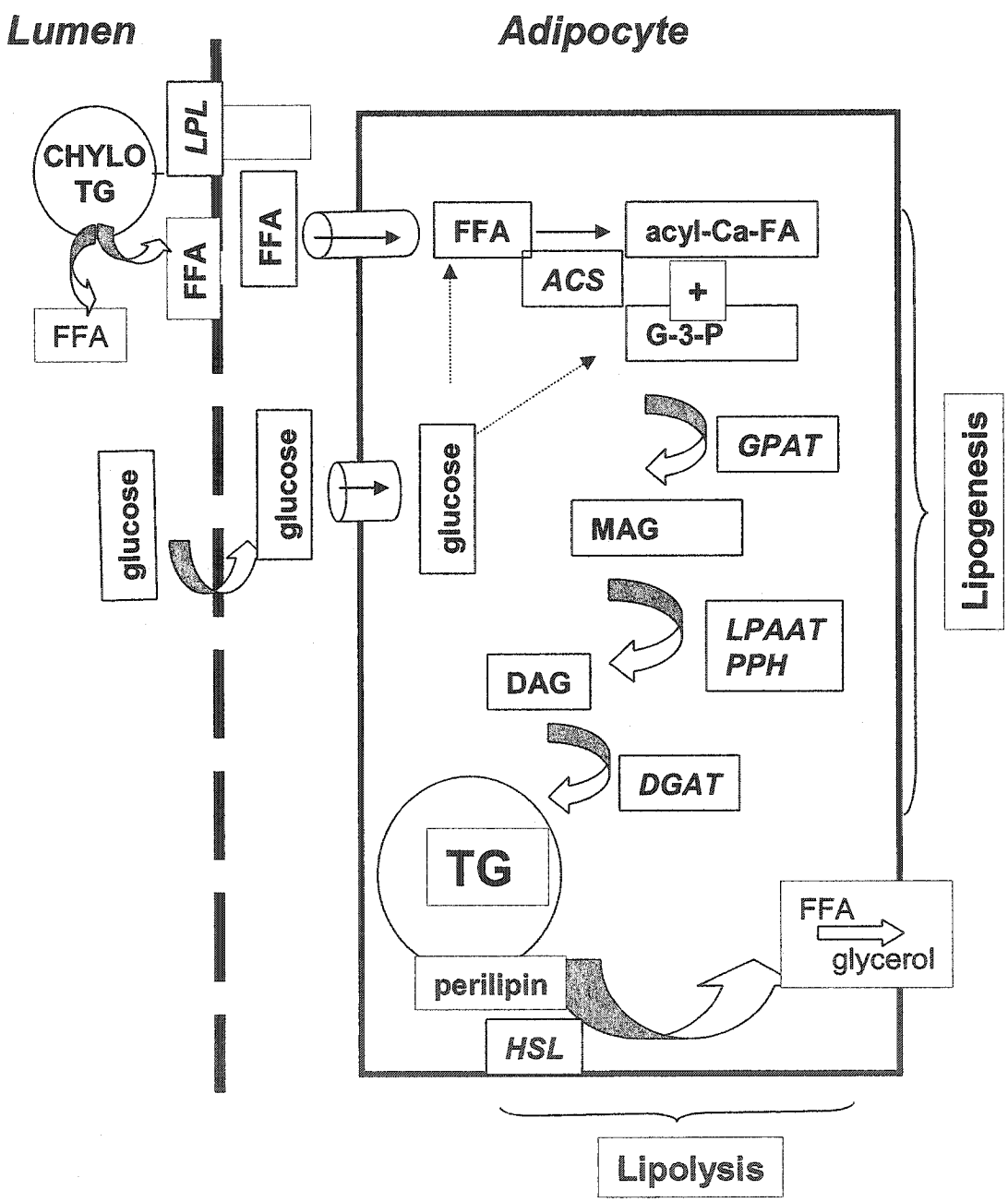

Lumen
Adipocyte
CHYLO TG
LPL
FFA
FFA
FFA
FFA
acyl-Ca-FA
ACS
+
G-3-P
glucose
glucose
glucose
GPAT
MAG
LPAAT
PPH
DAG
DGAT
TG
perilipin
HSL
FFA
glycerol
Lipogenesis
Lipolysis

Figure 1.1 Simplified Diagram of Intracellular Triglyceride Metabolism.

Shown are the basic steps involved in the biosynthesis of triglyceride molecule. Glucose and fatty acids are the building blocks for each of the steps of the pathway. G-3-P is provided by the glycolytic pathway (glucose) to which sequential esterification of acyl-CoA-FA (activated fatty acid) is catalyzed by the intracellular enzymes. Chylo (chylomicron lipoprotein particle), free fatty acids (FFA), glycerol 3-phospate (G-3-P), monoacylglycerol (MAG), diacylglycerol (DAG), triglyceride (TG); *enzymes:* lipoprotein lipase (LPL), acyl-CoA synthase (ACS), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidate phosphohydrolase (PPH), diacylglycerol acyltransferase (DGAT), hormone sensitive lipase (HSL).

supplied by chylomicron or VLDL from the diet or made *de novo* from available glucose.

1.1.2.1 Activation of free fatty acids

With respect to the initial fatty acid entry into the cell, two theories are presently held, one suggesting passive flip-flop and, the other facilitated plasma membrane transporter mediated diffusion (10;11). A number of FA transporters have been identified and various research groups are pursuing their role in FA transport using various methods and cell systems (12). One always has to keep in mind that both processes may in fact be at work but may predominate at different times such as fasting or fed state. Once inside the cell, a membrane bound enzyme acyl-CoA synthase (ACS) adds a CoA thioester to 10-20 carbon length fatty acids, irrespective of their fate (lipid synthesis, oxidation, elongation or desaturation) (13). However, evidence exists that different isoforms of ACS will activate fatty acids destined for different biosynthetic pathways such as those of TG or phospholipid (14).

1.1.2.2 Enzymes involved in triglyceride synthesis

Although little is known of hormonal regulators of TG synthesis in adipocytes and the intracellular signaling pathways involved, the main enzymes responsible for TG synthesis have been known for sometime. For example, some of those enzymes (diacylglycerol acyltransferase (DGAT) and glycerol-3-phosphate acyltransferase (GPAT)) have only recently been cloned (15), (16) and

DGAT (-/-) mice, characterized by reduced fat mass, have just been generated (17).

Once the fatty acids are converted to fatty acyl-CoA form, they are rapidly incorporated onto a glycerol backbone one molecule at a time to form *de novo* TG molecules. GPAT is the initial enzyme catalyzing the incorporation of the fatty-acylCoA onto glycerol-3-phosphate to generate 1-acyl-glycerol-3-phosphate (also known as lysophosphatidic acid) (18). The activity of GPAT is tremendously increased during differentiation of 3T3-L1 preadipocytes into adipocytes (19). The acylation of the second fatty-acylCoA group onto the 1-acyl-glycerol-3-phosphate is catalyzed by lysophosphatidic acid acyltransferase (LPAAT) of which a number of cell type specific isoforms have been characterized (20;21). The resulting molecule is known as phosphatidic acid and the activity of LPAAT is similarly augmented during the differentiation process (22). Both GPAT and LPAAT are found mainly in the microsomal fraction and the products generated by their action can enter not only TG synthetic pathway but also that of phospholipids. The formation of diacylglycerol is subsequent to the removal of the phosphate group by phosphatidate phosphohydrolase (PPH-1). Although little is known about this enzyme, it has been suggested to be an important regulatory enzyme since hormone-induced changes in its activity correlate significantly with the rate of TG synthesis (23). Perhaps the only enzyme unique to the TG synthetic pathway is DGAT. This microsomal enzyme catalyses the final, and believed to be rate limiting, step in TG formation. Interestingly, the increase in the mRNA levels of DGAT during adipocyte differentiation does not match the

enormous increases in its activity, indicating a possible regulation by posttranscriptional modification (22;24).

1.1.2.3 Regulation of glucose entry

Glucose is a substrate for both the glycerol-3-phosphate (G-3-P) backbone as well as *de novo* fatty acid synthesis. Glucose entry into the cells is mediated by various cell type-specific glucose transporters (Glut), which in turn are under hormonal control and dependent on extracellular concentration of glucose. In adipocytes and muscle, there are two kinds of glucose transporters Glut 1 and Glut 4. Glut 1 is present at the cell membrane at all times whereas Glut 4 can be recruited from the intracellular depot by insulin in response to raising glucose levels (25;26). Upon entry, glucose is trapped inside the cell by the phosphorylating action of hexokinase. Subsequently, glucose entry into the glycolytic pathway yields dihydroxyacetone phosphate (DHP) and glycerolaldehyde 3-phosphate (GAP) by the ordered action of numerous enzymes. At this point, DHP can either follow two fates: 1) it can be converted to GAP and further broken down to generate energy or 2) it can be converted to G-3-P and utilized as a backbone for either TG or phospholipid (as described above). With respect to *de novo* fatty acid synthesis, it is low in adipose tissue when the supply of exogenous FA is satisfactory (27).

1.1.3 Lipolysis

In response to energy demands, the FA of stored TG can be mobilized for use by peripheral tissues. The release of metabolic energy (FA), during the process of lipolysis, is controlled by stimulation of a complex intracellular cascade resulting in the activation of hormone sensitive lipase (HSL) (28). In adipocytes, the activation of this process is brought about by hormones such as glucagon or epinephrine, which upon binding to their respective receptors activate adenylate cyclase (29). This in turn increases cAMP levels activating protein kinase A (PKA), which subsequently, through phosphorylation, activates HSL (30;31). Upon activation, HSL translocates from the cytoplasm to the surface of the intracellular lipid droplet and hydrolyzes intracellular TG to generate its primary building blocks: FA and glycerol. A recently identified protein, perilipin, was shown to regulate HSL access to the lipid droplets, such that in perilipin knockout (KO) mice HSL is very active and the mice are lean (32;33). Insulin has been shown to inhibit lipolysis by inhibition of HSL. Insulin activates phosphodiesterase (responsible for cAMP breakdown) which results in HSL dephosphorylation and, ultimately, its inactivation (34).

1.2 Adipogenesis

Excess fat mass, characteristic of the obese state, is brought about by increases in adipocyte size and by recruitment of fat cell precursors, preadipocytes, to become adipocytes (adipogenesis). Adipogenesis (see Figure 1.2) is a result of transcriptional activation of various genes in a well-defined sequence and the first step of this process is characterized by growth-arrest phase. In the *in vitro*

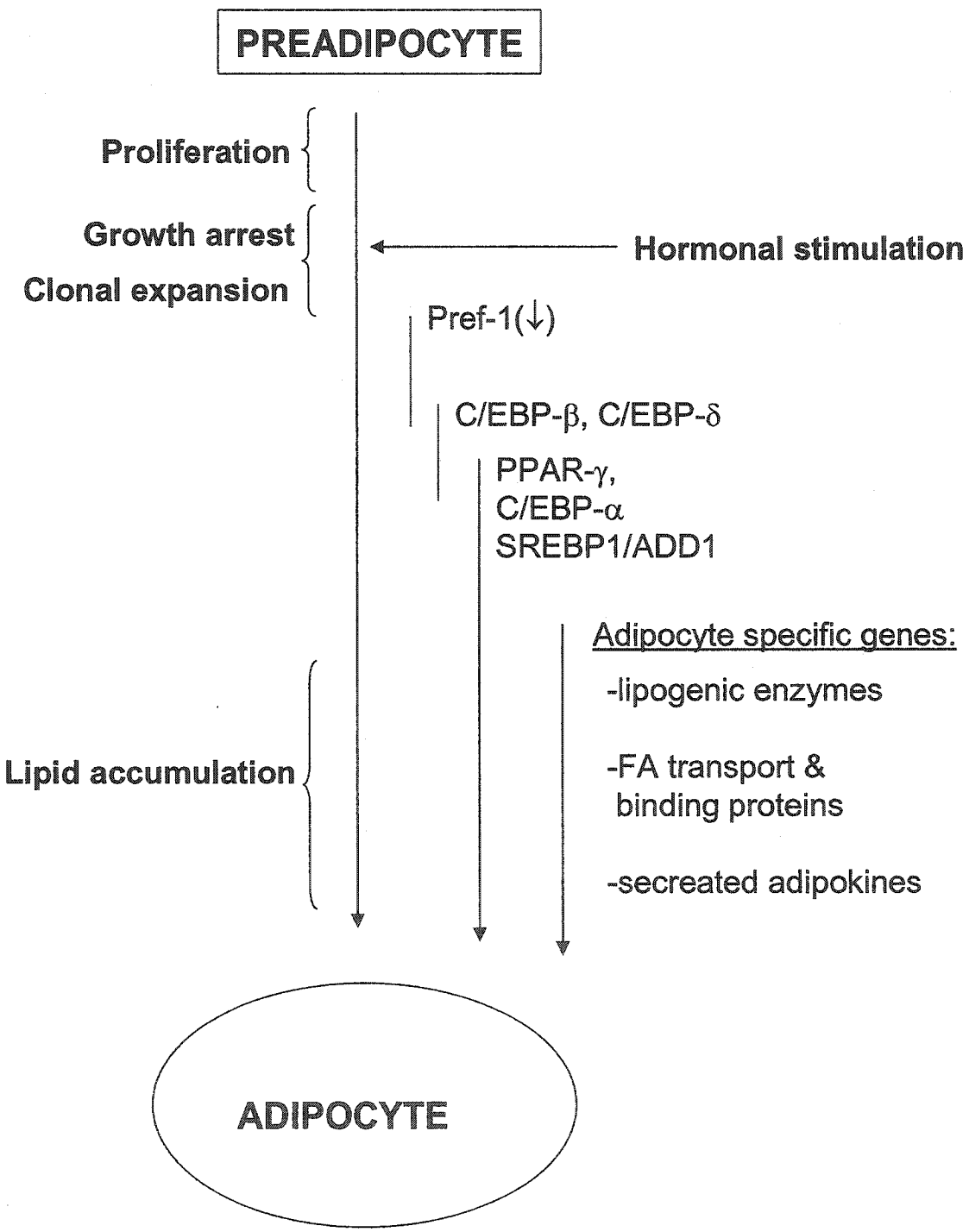

PREADIPOCYTE
Proliferation
Growth arrest
Clonal expansion
Hormonal stimulation
Pref-1(↓)
C/EBP-β, C/EBP-δ
PPAR-γ,
C/EBP-α
SREBP1/ADD1
Adipocyte specific genes:
-lipogenic enzymes
-FA transport &
binding proteins
-secreated adipokines
Lipid accumulation
ADIPOCYTE

Figure 1.2 Schematic Representation of Basic Stages and Events of Adipogenesis (Adipocyte Differentiation).

The main stages of adipogenesis are proliferation, growth arrest and lipid accumulation (shown to the left). Given appropriate hormonal stimulation, during a defined time period, preadipocytes will exit cell cycle and enter the process of differentiation to become adipocytes. The molecular events along with their approximate duration are shown on the right. Preadipocyte factor-1 (pref-1), CCAAT/enhancer binding protein (C/EBP), peroxisome proliferator-activated receptor (PPAR), sterol regulatory element binding protein-1 (SREBP1)/adipocyte determination and differentiation factor-1 (ADD1).

systems, this is accomplished through contact inhibition, although some experimental data does not support that hypothesis (35).

Following growth arrest, the cells must receive appropriate hormonal stimuli. In tissue culture this is dependent on the specific cell culture model used such as, preadipocyte cell lines *vs.* primary preadipocytes or mouse *vs.* human cells (35). The standard adipogenic hormonal cocktail contains a glucocorticoid (dexamethasone), cAMP stimulatory agent (isobutylmethylxanthine) and supraphysiological concentrations of insulin. In contrast, in an *in vivo* situation, the adipocyte-specific hormonal signal which is received by the preadipocytes, that result in adipogenesis is not as yet well understood although a number of potential candidates are being investigated (discussed below). Post-confluent preadipocytes undergo at least one round of DNA replication and cell doubling, leading to the clonal amplification of committed cells (36). This is not necessarily true for human primary preadipocytes (37).

The first hallmark of the adipogenesis process is the change in cell morphology, paralleled by the changes in extracellular matrix and cytoskeletal components (38). Most of all, the changes that occur take place at the gene expression level, in that investigators now characterize them into early, intermediate and late marker category (39). Exposure of confluent preadipocytes to the adipogenic cocktail induces expression of C/EBP-β and C/EBP-δ which in turn activate PPAR-γ and C/EBP-α, however, the nature of endogenous ligands and the molecular mechanisms responsible for their production are not well characterized (40). In addition, transcriptional regulators such as ADD/SERBP-1

(41), GATA-binding transcription factors (42) and CREB (43) also play a critical role in the molecular control of the process of preadipocyte to adipocyte conversion as demonstrated by numerous *in vitro* overexpression or knockout systems. During the terminal phase of differentiation, activation of the transcriptional cascade leads to increased mRNA, protein and activity levels of the enzymes involved in TG synthesis (ACS, GPAT, DGAT, adipsin). The mature adipocytes are now competent to not only regulate their lipid metabolism at the intracellular, enzymatic level but they also gain the capacity to secrete various peptides, proteins, hormones and cytokines (collectively known as adipokines; for review see (44)).

1.3 Adipose Tissue as an endocrine organ

It is now well established that adipose tissue, long regarded as a storage organ, is actively involved in the production of adipokines that can regulate a number of metabolic processes in an endocrine, paracrine or autocrine manner. Their primary roles range from effects on blood pressure (angiotensin), to energy metabolism (leptin, adiponectin) all the way to those whose roles are not yet well-understood (resistin). While it is well known that circulating hormones produced elsewhere such as glucocorticoids (adrenal cortex), catecholamines (sympathetic nervous system adrenal medulla), sex hormones (gonads) and insulin (pancreas) affect lipogenesis, lipolysis and adipogenesis (29;45), there is increasing evidence that hormones secreted specifically by adipocytes may play as important a role. Of the many factors secreted by the adipose tissue only a few

have been proposed to directly influence lipogenesis, lipolysis or adipogenesis through the regulation of processes such as *de novo* fatty acid synthesis, glucose transport, triglyceride synthesis, lipolysis and differentiation (described below).

1.3.1 Anti-adipogenic factors

Numerous studies using various experimental models have demonstrated that leptin, TNF-a (tumor necrosis factor), TGF-ß (transforming growth factor) and interleukins influence lipolysis and inhibit adipogenesis.

Although, leptin effects are mainly endocrine in nature (hypothalamic control of food intake and energy expenditure) (46), the recent discovery of specific leptin receptor isoforms on adipocytes (47) indicated that leptin may have peripheral effects as well. In mice, majority of reports suggest that leptin decreases lipogenesis (48), increases lipolysis (49;50), counteracts the inhibitory effect of both insulin (51) and adenosine (52) and prevents lipotoxicity. However, establishing the specificity of leptin action in human adipocytes needs further investigation (53).

TNF- α has been long recognized as a regulator of fat cell function although recognition that it is produced by adipocytes is recent (54). In preadipocytes, it effectively inhibits differentiation as demonstrated by reduced lipid accumulation and decreased gene expression of LPL, glycerolphosphate dehydrogenase (GPDH), Glut 4 and fatty acid binding protein (54;55). In differentiated adipocytes, TNF-α is a potent inhibitor of lipogenesis, and a potent stimulator of lipolysis. Lipolytic stimulation is evidenced by increased glycerol

release (56;57) mediated by increased HSL activity (58). In addition, not only does TNF-α inhibit differentiation through disruption of mitotic clonal expansion (59) but it can also reverse adipogenesis (60). These effects are believed to involve TNF receptor 1 and not TNF receptor 2 (61). Most importantly TNF-α interferes with insulin signaling, rendering cells insulin resistant (62;63).

A number of interleukins have also been shown to be secreted by adipocytes and several of them are involved in decreasing or increasing lipolysis. IL-6, the best characterized cytokine in terms of its effects on adipose tissue, is increased in obesity and diabetes and is associated with insulin resistance (64;65). TGF-β, on the other hand, has been shown to inhibit preadipocyte differentiation *in vitro* (66), although the specific mechanism of this inhibition is not fully understood. Finally, preliminary studies implicate increased pref-1 expression in the inhibition of differentiation and understanding its role in the regulation of adipose tissue physiology is currently subject of intensive research (67).

1.3.2 Pro-adipogenic factors

The list of the factors secreted by adipose tissue, that are implicated in regulation of lipogenesis or adipogenesis is even shorter. Presently, angiotensin II, prostaglandins and agouti are believed to be pro-adipogenic with acylation stimulating protein (ASP) being the most potent of them. Especially interesting is the fact that observations in human and murine experimental models (*in vitro* and *in vivo*) with respect to the action of ASP are concordant.

Angiotensinogen and the complete renin-angiotensin system necessary for angiotensin II production exist in adipocytes (68). Angiotensin II stimulation of TG synthesis has been demonstrated in 3T3-L1 cells, however the effects were small (30%) and only observed after 48-hour incubation period (69). These observations were extended further to show that this effect is most probably due to induction of prostacyclin (PGI_2) release and its action on adipocytes (70;71). The fact that angiotensin-deficient mice have reduced adipose tissue mass further implicates this hormone in regulating adipose tissue energy stores (72). Differential expression of angiotensin II receptors during the differentiation process also points to its possible involvement in the process of adipogenesis (73). Agouti and its human homologue, agouti-signaling protein exert their action by interacting with specific melanocortin receptors also present on adipocytes (74;75). In mice, dominant mutations in agouti gene result in ubiquitous expression of its product (including adipose tissue) and are characterized by obesity and insulin resistance (76;77). Moreover, agouti increases TG content in 3T3-L1 adipocytes (78). Recent data indicates that, at least in rodents, agouti action on adipogenesis is achieved through the regulation if intracellular calcium levels. In humans, although agouti gene is mainly expressed in adipose tissue (79) and increases during differentiation process (80) its molecular effects are not yet clear.

Acylation Stimulating Protein Role in Lipid Metabolism

1.4 Acylation Stimulating Protein

As early as 1987, Cianflone and Sniderman conducted the first studies identifying an acylation stimulating activity in plasma. Originally, all that was known was that a protein component detected in circulating blood was responsible for the increased intracellular TG esterification rates in human skin fibroblasts (81). Subsequently, the identity of that protein fraction, which was named based on its function - Acylation Stimulating Protein (ASP), was established to be identical to C3a desArg (82).

1.4.1 Mechanism of ASP (C3a desArg) generation

ASP is a 76 amino acid (8932 kD) fragment generated by the ordered interaction of complement C3, Factor B and adipsin, all belonging to the alternative complement pathway (Figure 1.3) (83). The activation of this pathway starts by the interaction of the C3b component of C3 (ASP precursor) with the plasma membrane. In the presence of Mg^{2+}, Factor B binds to the activated C3b to form a C3bB complex. This, in turn, induces conformational change of the Factor B and permitting proteolytic cleavage by adipsin (a serine protease enzyme). The action of adipsin (also known as complement Factor D) C3b-bound Factor B generates C3bBb fragment. C3bBb is an enzyme, C3 convertase, which in turn cleaves complement C3 into C3b and C3a (84;85). The C3b component can then be recycled back to start the process anew. C3a, on the other hand, is rapidly converted in plasma by carboxypeptidase B to form C3a desArg (ASP)

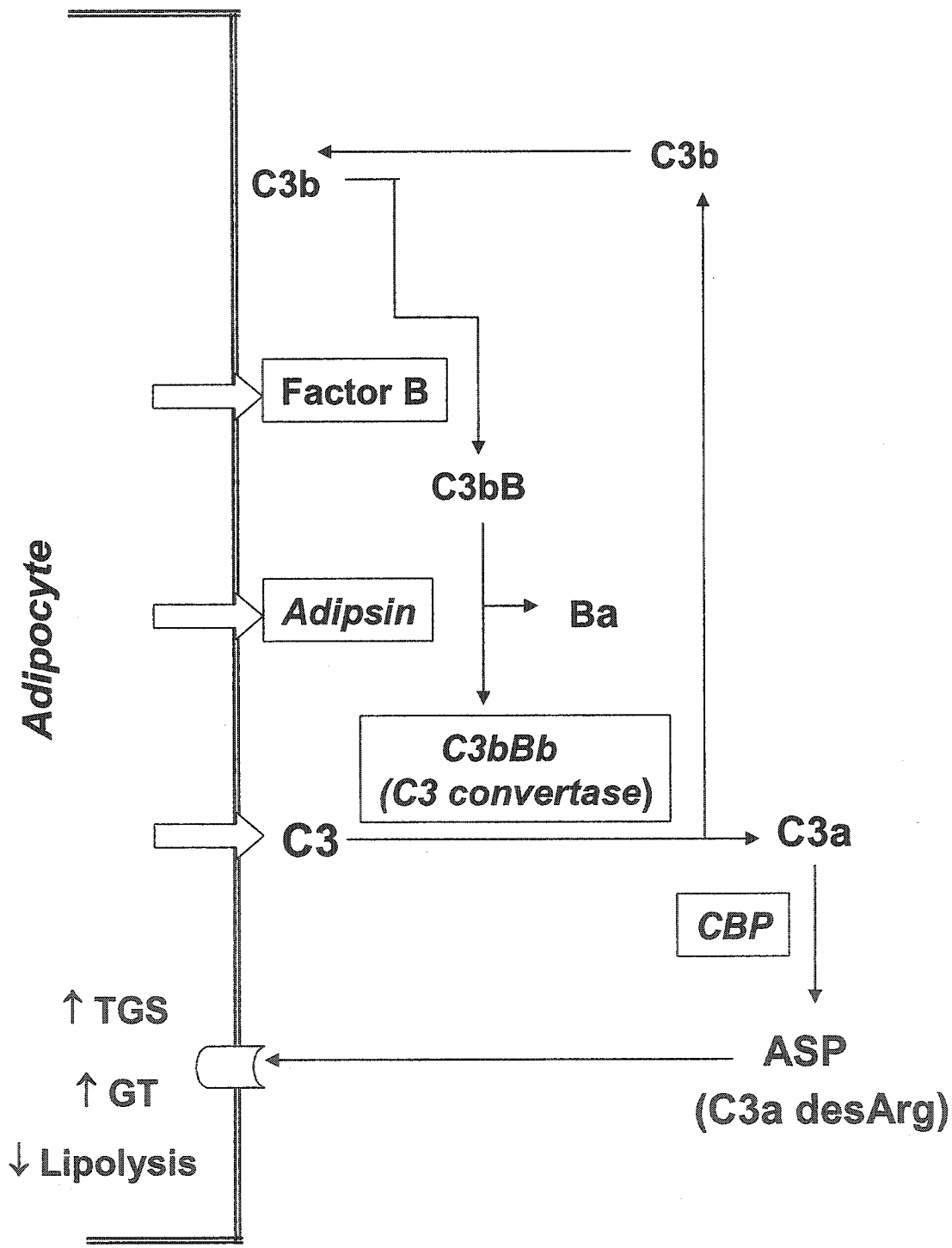

C3b
C3b
Factor B
C3bB
Adipocyte
Adipsin
Ba
C3bBb
(C3 convertase)
C3
C3a
CBP
↑ TGS
↑ GT
↓ Lipolysis
ASP
(C3a desArg)

Figure 1.3 Diagram of ASP generation.

The interaction of Factor B, adipsin and complement C3 results in generation of ASP (C3a desArg) by the adipose tissue. All proteins are produced by adipocytes in a differentiation dependent manner. Generated ASP is now available to interact with the cell membrane receptor in an autocrine/paracrine manner. Increased triglyceride synthesis, increased glucose transport and decreased lipolysis are the results of ASP action. Acylation stimulating protein (ASP), carboxypeptidase (CBP), triglyceride synthesis (TGS), glucose transport (GT).

(86). Interestingly, the other components of the alternative complement pathway such as C5-C9 are never activated (87), and thus, cell lysis does not take place nor is generation of ASP is a byproduct of an immunologic response. Murine and human adipocytes synthesize and secrete all three proteins (C3, Factor B and adipsin) of the alternative complement pathway but not C2 and C4 (components of the classical complement pathway) (87-89). Moreover, *in vitro* studies provided conclusive evidence that C3a (in test tube reconstitution experiments, (82)) and C3a desArg (recombinant production of C3a desArg, (90)) possess the same acylation-stimulatory activity as that observed for plasma isolated ASP.

1.4.2 ASP production *in vitro* and *in vivo*

Plasma ASP concentration in a general population is on average 25 nM with no difference between non-obese man and woman (91). In obesity, one sees quite a substantial increase in the circulating ASP levels (91), that decrease with weight loss (92;93). One possible explanation is that the increased fat mass in morbidly obese subjects is responsible for this increase. Using human cultured adipocytes as a model, Cianflone *et al* demonstrated that ASP production is increased during preadipocyte to adipocyte differentiation process resulting in many fold greater levels of ASP in adipocytes (89). In addition, the expression levels of the 3 proteins involved in ASP generation (C3, Factor B and adipsin) are increased over the same time period. In the postprandial period ASP levels in the general circulation usually do not change with occasionally a slight decrease over the 6-8 hour period (94). This implies that perhaps changes in ASP levels maybe

taking place within the microenvironment of the adipose tissue and this was addressed by Saleh *et al* who determined that adipose tissue specific ASP production takes place following the meal (95). In the elegant series of experiments looking at the arterial-venous differences across a subcutaneous adipose tissue bed, they have shown ASP increases in the venous blood draining adipose tissue. This significant raise in ASP concentration, which reflects the microenvironment of the adipose tissue, was coincided with the increased plasma TG clearance. *In vitro* studies have further shown that the chylomicron (dietary fat) lipoprotein fraction is in fact responsible for the increased ASP (and precursor C3) production from the adipose tissue although there was a moderate effect of insulin as well (96). The specific component of the chylomicron particle was subsequently isolated and identified to be transthyretin (TTR) (97), that together with retinoic acid (98) are the possible agents responsible for increased ASP and C3 levels.

1.4.3 ASP function

Until recently insulin was believed to be the only hormone that upregulates TGS in adipocytes. These effects are known to involve increasing in LPL activity (99) stimulation of glucose transport (100) and inhibition of the lipolytic action of HSL (101). ASP, however, has been proven to markedly stimulate TG synthesis in a number of cell lines from mouse and human (102;103). The increased rates of fatty acid incorporation into TG (FA esterification) by ASP have been shown to be much higher in human adipocytes than preadipocytes or fibroblasts (82). In a

majority of studies, however, the effects of ASP are studied on human skin fibroblasts since they provide not only an excellent and reproducible cell model but also are representative of a physiological state of the subjects. At the present time ASP effects are believed to involve, in particular, activation of DGAT, the final enzyme of the TG pathway (see Figure 1.1) (104). Since the analysis of the enzyme kinetics (true for both TGS or glucose transport) show that the ASP effect is predominantly on V_{max} (enzyme activity) and not K_m (substrate presentation) (105), Yasruel *et al* examined ASP effects on the individual enzymes of the TG synthetic pathway (104). Using crude microsomal preparations, they have shown that ASP markedly activated DGAT (2-fold increase in activity) whereas GPAT activity increased only 23%. In contrast, ASP had no effect on either ACS or PPH-1 activity.

Additional experimental data indicated that ASP also stimulates glucose transport in human skin fibroblasts (105), human adipocytes (106) and myotubes (107). Translocation of specific glucose transporters (Glut 4, Glut 3) from the intracellular pool to the plasma membrane was reported as well in response to ASP (105;107). Although ASP also stimulates glucose transport, the effect of ASP on TG synthesis is not solely mediated through an effect on glucose transport since substitution of pyruvate (alternative source of G-3-P) for glucose does not abolish ASP effects.

Recently, the work of Van Harmelen *et al* established that ASP action is not limited to stimulatory effects on TGS or glucose transport but also on the inhibition of lipolysis in human adipocytes (108). ASP inhibits basal and

norepinephrine induced FFA release by predominantly stimulating phosphodiesterase 3 and 4. Interestingly, although ASP effects are comparable in magnitude to those of insulin (TGS, glucose transport and lipolysis) they are additive.

1.4.4 ASP signaling pathway specific to TGS

To date, little is known of the various intracellular players involved in ASP action. Dependence on protein kinase C (PKC) but not PKA activation was clearly demonstrated in the work by Baldo *et al* (109). Using HSF as cell model, time-dependent translocation of PKC from the cytosolic pool to the membrane fraction has been shown upon addition of ASP. This was supported by inhibition of ASP stimulated TGS through various PKC inhibitors (staurosporine, GF109203X and calphostin C). To support data on PKC activation, intracellular diacylglycerol (stimulatory lipid component for the majority of PKC isoforms) increases were evident as two peaks at 10 and 30 minutes post ASP treatment. This biphasic pattern of DAG generation suggests that breakdown of different phospholipids is involved, therefore, pointing to possible involvement of phospholipases (PLC and/or PLD).

1.4.5 Lessons from C3 (-/-) (ASP deficient) mice

Recent publications in a C3 KO mouse model support the role of the ASP pathway *in vivo*. Murray *et al* demonstrated that ASP deficient male mice (C3 KO) manifested a delay in TG clearance, which was reversed upon

intraperitoneal ASP injection (110;111). Similar trend was observed in two models of mouse obesity (*ob/ob* and *db/db*). The changes, however, were more pronounced, comparable between both mouse strains and included significant decreases in postprandial glucose and non-esterified fatty acid levels (112).

Furthermore, C3 (-/-) had reduced body weight, compared to wild type, due to the substantial reduction in white adipose tissue mass (50%), especially evident in females (113). Increase in insulin sensitivity was also observed. Studies on a single knockout were further extended to a C3 knockout on the obese background (*ob/ob*, mice deficient in leptin) (114). It was hypothesized that the resulting ASP deficiency on the obese background (2KO) could have a protective function. Not only did the 2KO have delayed TG and FA clearance as compared to *ob/ob* mice (and similar to C3 KO), they were also characterized by reduced body weight due to adipose tissue reduction. Surprisingly, food consumption was increased and this was balanced by a significant increase in energy expenditure. In addition, just as seen in C3 -/- mice, an increase in insulin sensitivity in the double knockout mice as compared to *ob/ob* was demonstrated. The close correlation between the body weight and HOMA (the homeostasis model assessment for insulin resistance) index suggests that it is the decreased weight that results in increased insulin sensitivity. This study provides yet another example of *in vivo* evidence for the importance of ASP in the regulation of lipid metabolism and adipose tissue mass.

1.4.6 Evidence for the ASP receptor

Although the precise mechanism of ASP action is not yet fully understood it is believed the effects of ASP are the result of its interaction with a specific cell surface receptor. Detailed structure-function studies by Murray *et al* demonstrated that modified ASP, which cannot bind, is not active (115). On the other hand, certain modifications, which destroy ASP activity, do not affect the cellular binding. Disruption of disulfide-linked core region or basic charges in C-terminal region of ASP result in abolishment of TG stimulatory activity but maintain binding of ASP to the receptor. Based on these results, it was concluded that the binding region is different from the region involved in stimulating TGS. Using radiolabelled ASP a number of studies demonstrated that ASP binding is saturable and specific in human adipocytes, preadipocytes and fibroblasts (115-117). The binding affinity is high with K_D values ranging from 25 to 150 nM and cannot be competed out by a similar size and charge molecule (cytochrome C). In a study by Zhang *et al*, where cellular responses of cultured HSF from normal and hyperapoB subjects were examined (117), ASP bound with high affinity to normal HSF. Cells with decreased ASP response to TGS and glucose transport also evidenced proportionally decreased ASP binding. Moreover, ASP binding to human adipocyte plasma membranes was shown to be influenced by depot site and gender. Binding in membranes from subcutaneous adipose tissue was greater than in omental adipose tissue and the binding affinity was higher in females as compared to males (116). All these results point to a presence of specific high affinity ASP receptor in adipocytes.

1.4.6.1 Complement C3a and C3a receptor

As noted above, structural characterization established that ASP is identical to C3adesArg (82). C3a (the immediate ASP precursor) is a small, basic polypeptide and the terminal arginine has been shown to be essential for both binding and biological activity (118;119). In the immune system, C3adesArg, on the other hand, has been generally accepted as an inactive by-product of complement activation, although a few recent reports demonstrate that this may not be the case (120;121). Amongst its *in vitro* effects, C3a has been shown to stimulate release of lysosomal enzymes (122;123), and increase vascular permeability (124) and smooth muscle contraction (125). In a number of studies where the activity of both C3a and C3adesArg was tested only C3a, but not C3adesArg, stimulated guinea pig platelet activation (126), induced chemotaxis of human mast cells (127) and macrophages (128), and influenced cellular calcium release (127;129). In the few studies where both C3a and C3adesArg were active, such as modulation of cytokine levels in B cells (120) and peripheral blood mononuclear cells (121), the target cells had to be specifically pretreated with such agents as Il-1 or LPS. *In vivo*, plasma levels of C3a have been shown to increase in a number of inflammatory disease states such as systemic lupus erythematosus (130), rheumatoid arthritis (131) and in sepsis (132).

C3a, as well as another complement protein such as C5a, elicit their effects by binding to specific cell surface receptors (133;134). Recently, both the human and mouse C3a receptors have been cloned and characterized (135-

137). The C3a receptor, a seven transmembrane G-protein coupled receptor, has been studied primarily in myeloid cells (macrophages, mast cells, platelets), but recently its existence has also been also demonstrated on non-myeloid cells (lung, heart, brain,) (138-140). The affinity (K_d) of the C3a receptor varies from 0.8 to 200 nM depending on the cell type, species and experimental procedure (136;140-142). This wide range is also true with respect to receptor number. Binding studies with both C3a and C3adesArg on guinea pig platelets (138), human mast cells (127) and macrophages (119) have demonstrated that, in contrast to C3a, C3adesArg (ASP) does not bind to these cells. Furthermore, ASP does not bind to cells transfected with C3a receptor. Those studies that actually demonstrated a C3a-like activity for C3adesArg either did not do simultaneous binding studies (127;143) or implicated a non-specific interaction with an unidentified component of cell membrane (120;143;144). Thus, the mechanisms for activation by C3a desArg are not clear.

Considering the structural similarities of ASP and C3a it is surprising to see such diverse physiological responses, which appear to be tissue and cell type specific. The nature of the activities exhibited both *in vitro* and *in vivo* by ASP strongly implies that they are the result of ASP interaction with a specific cell surface receptor different from the C3a receptor.

1.5 Scientific rationale and objectives

The ASP pathway plays an important role in regulating lipid metabolism as has been demonstrated by both *in vitro* and *in vivo* data. Disregulation of this

pathway, whether it is at the hormone (ASP) or the receptor level, may be implicated in obesity. The goal of this thesis is to further our understanding of the ASP pathway in the regulation of lipid metabolism at the cellular level and this was done by investigating the following:

1) To see if, in addition to the acute actions of ASP on TG synthesis, ASP participate in induction of adipocyte differentiation.
2) To identify the ASP receptor.
3) To further analyze the signaling components of the ASP pathway.

CHAPTER 2

Evidence for a Novel Role for Acylation Stimulating Protein (ASP): an *in vitro* Regulator of Adipocyte Differentiation

Magdalena Maslowska[1], Hong Wei Wang[1], Rob Sladek[2], Katherine Cianflone[1]

[1]Mike Rosenbloom Laboratory for Cardiovascular Research and [2]Montreal Genome Center, McGill University Health Centre, Montreal, Quebec, Canada.

Short Title: ASP Induces Adipocyte Differentiation

Key Words: microarray, gene expression, C3a desArg, 3T3-F442A, 3T3-L1, triglyceride synthesis

Acknowledgements: This work was supported by grants from the Canadian Institute for Health Research to Katherine Cianflone. Hong Wei Wang is presently located at Tongji Medical University, Wuhan, China.

Submitted for publication Endocrinology, 2003 (revisions requested)

2.1 ABSTRACT

A number of adipose tissue produced factors have been shown to directly or indirectly participate in the control of energy storage with the potential for a role in obesity. Acylation stimulating protein (ASP), a protein produced by adipose tissue, effectively stimulates triglyceride synthesis (TGS) and glucose transport and inhibits lipolysis. The significant decreases in adipose tissue mass in ASP deficient mice and the recent identification of a cell surface receptor for ASP in adipocytes, prompted us to investigate the ability of ASP to induce differentiation. In 3T3-F442A cells, incubation with ASP alone induced differentiation as determined by Oil-Red O staining and caused significant increases in TG synthesis and TG mass accumulation ($p<0.05$). ASP also induced clonal expansion with significant increases in DNA synthesis at 24 hrs as compared to non-differentiating NIH-3T3 or untreated cells ($p<0.003$). Parallel changes were observed in 3T3-L1 cells and the effects were comparable to those observed for insulin. Gene expression of transcriptional regulators (C/EBP-d and PPAR-?) and genes involved in lipid metabolism (diacylglycerol acyltransferase and adipsin) during differentiation demonstrated similarities between ASP and insulin treatment. Finally, we analyzed microarray gene expression in 3T3-F442A cells treated with either ASP or insulin during the very early time points of differentiation (1, 3 and 24 hours). The genes induced and suppressed with ASP are substantially different from those of insulin. Thus, our study demonstrates that, when substituted for insulin, ASP can induce

differentiation in murine 3T3-F442A and 3T3-L1 preadipocyte cell lines, potentially through an alternate mechanism than insulin.

2.2 INTRODUCTION

In the last decade obesity has become a major health focus due to its increasing prevalence (1,2). The consequences of obesity extend to health problems such as diabetes, cardiovascular disease, infertility, immunological perturbances and others (3-5). Adipose tissue provides the means by which the body regulates energy requirements by controlled storage and release of fatty acids, in that, not only do adipocytes have the capacity to adjust their triacylglycerol (TG) store size, but preadipocytes have the potential to differentiate.

The impact of adipocyte derived factors on both adipocyte differentiation and development of obesity has attracted increasing attention (6). In that respect, TNF-a, IL-6, pref-1 and resistin are all proposed to either inhibit differentiation or induce de-differentiation (7-10). On the other hand, only prostaglandins and their derivatives (also produced by adipose cells and act *via* PPAR-?) have been implicated in induction of differentiation (11). In light of the recent identification of a cell surface receptor for ASP in adipocytes (12) and a reduced adipose tissue mass in C3 (ASP deficient) knockout mice (13-16), the present study was undertaken to see if ASP, an adipokine, has the potential to induce differentiation.

ASP directly influences cellular lipid metabolism through TG synthesis stimulation, increased glucose transport, and inhibition of lipolysis in a variety of cells from different species (17). In 3T3 cells specifically, significant increases in TG synthesis and glucose transport have been demonstrated for ASP as well as

its precursor C3a (18,19). In this regard, the effects of ASP are comparable and additive to insulin, however, preliminary evidence suggests different intracellular pathways (20). Whereas insulin acts mainly as an endocrine factor, it is thought that ASP action is primarily autocrine/paracrine in nature. During differentiation, ASP production is increased (21) with adipocytes being more responsive to ASP stimulatory effects than preadipocytes for both TG synthesis (22) and glucose transport (23). Although circulating levels of plasma ASP do not change in the postprandial period, increased plasma ASP levels have been positively correlated with obesity, diabetes and cardiovascular disease (24).

In vivo human and mouse studies provide additional evidence for a potential role of ASP in the postprandial TG clearance and regulation of fat stores. Postprandially in humans, Saleh *et al* demonstrated increased adipose tissue production of ASP (venous-arterial differences), which was concurrent with increased plasma TG clearance (25). Chylomicrons (dietary fat), not free fatty acids, provide a triggering signal to increase ASP production in cultured human adipocytes (26,27). C3 (-/-) knockout mice, which are obligate ASP deficient, manifest significantly diminished adipose tissue mass (15,16) as do *ob/ob/* C3 *(-/-)* double knockout mice (14). The decreased adipose tissue mass contrasts with increases in food intake in both C3 (-/-) and *ob/ob/* C3 *(-/-)* mice, consistent with changes in energy expenditure. Also, both knockout mouse models have delayed postprandial plasma TG clearance, a consequence that is corrected by the intraperitoneal injection of ASP (14,15). These marked changes in adipose tissue mass raise the possibility that ASP action on adipose tissue may be mediated in

two ways. First, ASP can acutely influence plasma postprandial clearance of TG *via* its effects on adipose tissue storage and second, ASP may participate in the recruitment of preadipocytes to become adipocytes.

In vitro, the process of differentiation is triggered by insulin and glucocorticoids and 3T3 cells provide the most studied *in vitro* cell model to date. The pattern of gene expression is tightly controlled, initiated with a cascade-like activation of transcriptional factors and nuclear hormone receptors leading to the modulation of the expression of other genes. C/EBP-d, -ß and -a (CCAAT/enhancer binding proteins), ADD-1/SREBP-1 (adipocyte differentiation and determination factor/sterol regulatory element binding protein) and PPAR-? (peroxisome proliferator-activated receptor) are activated early and in a specific, well-defined sequence (28). These coordinated changes are, in turn, responsible for the induction of many genes including those involved in lipid metabolism (lipogenesis and lipolysis). For example, fatty acid synthase (29) and lipoprotein lipase (LPL) (30) are induced early while adipsin (31) and diacylglycerol acyltransferase (DGAT) (32) are induced late during differentiation. In this study, using 3T3 cells as a model, we have examined several stages of the differentiation process. We have demonstrated, for the first time, that ASP, an adipocyte produced hormone, has a potential to induce adipocyte differentiation and provide preliminary evidence suggesting that the ASP mechanism is different from insulin.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Materials

Dexamethasone, 1-methyl-3-isobutylxanthine, and insulin were purchased from Sigma (Oakville, Ontario, Canada). Tissue culture media (Dulbecco's minimum essential medium/Ham's F12 medium, DMEM/F12), phosphate buffered saline (PBS), and fetal bovine serum (FBS), TRIzol reagent and all RT-PCR reagents were from Gibco (Burlington, Ontario, Canada). All the organic solvents, general chemicals and tissue culture materials were purchased from VWR (Montreal, Quebec, Canada). Oleic acid [9,10 - ^{3}H(N)] (5.00 Ci/mmol) and thymidine [methyl - ^{3}H] (6.7 Ci/mmol) were purchased from DuPont - New England Nuclear (Mississauga, Ontario, Canada). Reagents for cell protein measurements were purchased from Bio-Rad (Mississauga, Ontario, Canada).

2.3.2 ASP preparation

ASP was isolated and purified from human plasma as described previously (33). Each batch was verified for purity by ion-spray mass spectrometry at McGill University Mass Spectrometry Unit. The activity of each ASP preparation was checked by its ability to stimulate triglyceride synthesis in 3T3-L1 preadipocytes.

2.3.3 3T3-L1 and 3T3-F442A cell culture and differentiation conditions

3T3-L1 cells were purchased from ATCC (Manassas, VA, USA). The cells were grown in DMEM/F12 media supplemented with 10% FBS (growth media) at 5% CO_2 to 70% confluency. For differentiation, cells were plated on 24-well plates at a cell density of $2x10^4$ cells/well. Growth media was changed every 2 days until the cells became confluent. To induce differentiation, two-day post-confluent cells (day 0) were supplemented with either 1 µM dexamethasone (DX) + 0.5 mM 1-methyl-3-isobutylxanthine (IBMX), DX + IBMX + 50 µg/ml ASP or DX + IBMX + 2 µg/ml insulin. Media was changed every two days until harvesting.

3T3-F442A cells were a kind gift of Dr. R. Germinario (Lady Davis Institute, McGill University, Montreal, Canada). The cells were grown in DMEM/F12 media supplemented with 10% FBS, 4.5 g/L glucose and 4 mM L-glutamine (growth media) at 7% CO_2 until 70% confluent. For differentiation, cells were plated on 24-well plates at a cell density of $2x10^4$ cells/well. Media was changed every 2 days until the cells became confluent. To induce differentiation, two-day post-confluent cells (day 0) were supplemented with 50 µg/ml ASP or 2 µg/ml insulin or both ASP and insulin. Media was changed every two days until harvesting.

2.3.4 TG synthesis and TG mass assay

The TG synthesis assay was similar to a previously published procedure by Baldo *et al* (33). 100 µM [^{3}H] oleic acid (average specific activity = 63 dpm/pmol) was complexed to bovine serum albumin (molar ratio 5:1) and added directly to the differentiation media) for the last 4 h prior to collection. Results are

expressed as pmol of [^{3}H] oleic acid incorporated into TG per µg of cell protein per 4 hours. The TG mass was measured by the method of Neri and Frings (34) and expressed as mg TG per mg cell protein.

2.3.5 RNA isolation and RT-PCR amplification

Differentiating cells were harvested at the indicated time points by removing the media and adding TRIzol reagent directly to the culture dishes. Total cellular RNA was isolated according to the manufacturer's instructions, quantified by spectrophotometry and its integrity was checked by a 1.5% agarose non-denaturing gel electrophoresis. To ensure good RNA recovery glycogen was routinely added to the isopropanol at the precipitation step. One well from a 6 well plate generated on average 30 µg of total RNA from 3T3-L1 and 42 µg from 3T3-F442A cells. Isolated RNA (3 µg) was denatured in the presence of RNase inhibitor (5 min, 65^{0}C) and reverse transcribed (RT) using a random hexamer primer (Gibco) to generate cDNA. The final RT mixture contained 1x buffer, 200 units of MMLV, 0.01 mM dithiothreitol and 0.5 mM of each dATP, dCTP, dGTP, and dTTP (2 hours, 37^{0}C). 4% of resulting cDNA was amplified by polymerase chain reaction (PCR) using primers designed in-house with a computer-assisted program (Primer 3, www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primer sets for murine DGAT, adipsin, C/EBP-d, PPAR-? were purchased from Gibco as presented in Table 1. For PCR, one cycle consisted of 1 min at 95^{0}C, 1 min at 60^{0}C and 1 min at 72^{0}C with 25 (DGAT and adipsin) and 30 (C/EBP-d, PPAR-?) cycles. The final PCR reaction mixture contained 1x buffer,

0.5 units of *Taq* polymerase, 0.2 mM dNTPs and 1 µM of each primer (5' and 3') in a final volume of 20 ul. For each of the primer products, multiple reactions were run to establish the optimal assay conditions for linearity and reproducibility and are presented in Table 1. 18S was used as the internal standard (housekeeping gene) as described by the supplier (Ambion, Texas, USA). Following PCR amplification, aliquots of PCR products were separated on a 7.5% polyacrylamide gel (using 0.8 % piperazine diacrylamide as crosslinker) with 100 base-pair DNA ladder (New England BioLabs) as standard. The gels were subsequently silver stained according to the manufacturer's instructions (Bio-Rad), scanned and analyzed using a densitometer (Molecular Analyst Program; BioRad, Ontario, Canada).

2.3.6 Clonal expansion and DNA synthesis (^{3}H Thymidine incorporation)

Clonal expansion was studied as [^{3}H] thymidine incorporation into newly synthesized DNA. The cells were plated, differentiation was initiated and the cells were harvested at 8 to 72 hours incubation. During the last 8 hours of incubation period, the differentiation media was spiked with [^{3}H] thymidine (2 µCi/ml) to measure clonal expansion. Cells were rinsed twice with cold PBS and precipitated overnight with 1 ml of 10% TCA. Cells were rinsed 3 times with 1 ml of ethanol, air-dried, and solubilized with 1 ml of 0.3 N NaOH for 30 min. Samples were neutralized (37 ul 4 N HCL) and used for radioactive counts and cell proteins (Bio-Rad protein assay) (35).

2.3.7 DNA microarray assay

Cells were harvested at 1 to 24 hours after initiation of differentiation. Total cellular RNA was then prepared by TRIzol extraction according to the manufacturer's instructions (as described above). To prepare probes for microarray analysis, 20 µg of total RNA was dissolved in DEPC-water, mixed with 100 pmol of T7- (T) 24 primer (Genosys, GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG- (T) 24) and this primer-RNA mixture was denatured for 10 minutes at 70°C and chilled on ice. First strand cDNA synthesis was performed using 2 ul of Superscipt II reverse Transcriptase (Gibco) in a 20 ul reaction mix (10 µM DTT, 500 µM each dNTP and First Strand Buffer (Gibco), 60 minutes, 42°C). Second strand synthesis was performed using 40 U DNA Polymerase I, 10 U E.coli DNA ligase, 2 U RNase H in 1x Second Strand Buffer (Gibco). The reaction was incubated at 16°C for two hours and stopped with 0.5 M EDTA. Following second strand synthesis, the cDNA probe was purified by phenol extraction and redissolved in DEPC water.

Biotinylated probe was prepared from the entire cDNA reaction using the ENZO Bioarray High Yield RNA Transcript labeling Kit (ENZO diagnostics). After reaction at 37°C for 5 hours with occasional agitation, the probe was purified using an RNeasy spin column (Qiagen), quantified by spectrophotometry and analyzed on a non-denaturing gel. The average probe length was reduced by incubating the purified probe in 1X Fragmentation Buffer for 35 minutes at 95°C. The hybridization mixture was prepared by mixing 15 µg of biotinylated probe with Control Oligonucleotide B2 (final concentration 50 pM) (Affimetrix), Herring

Sperm DNA (final concentration 0.1 mg/ml) (Research Genetics), Acetylated BSA (final concentration 0.5 mg/ml) (Gibco) in a final volume of 300 ul of 1x MES Hybridization Buffer (100 mM MES, 1 M NaCl, 20 mM EDTA, 0.01 % Tween-20) (all reagents Sigma). The hybridization mix was denatured for 10 minutes at 99^{0}C, 5 minutes at 45^{0}C and spun (5 minutes). The microassay was warmed up to room temperature and prehybridized in 1x hybridization buffer for 10-20 minutes at 45^{0}C. The prehybridization solution was removed and 150 ul of the hybridization mix was added to the array. The array and probe fragments were incubated at 45^{0}C overnight (16-20 hours). All experiments were performed using the GeneChip Mu1 1kSubB microarray (Affymetrix). This microarray contains approximately 6500 probe sets obtained mainly from the 3' regions of the corresponding cDNAs. When this array was designed, approximately 70% of the probes represented known genes, while 30 % were obtained from EST sequences. Following hybridization, non-specifically bound probe was removed by washing using GeneChip Fluidics Station 400 (Affymetrix). In total, ten low stringency washes (6x SSPE, 0.01% Tween-20, 0.005% Antifoam) and four high stringency washes (100 mM MES, 0.1 M NaCl, 0.01% Tween-20, 50^{0}C) were performed. Detection of specifically bound probe was performed by incubating the arrays with SAPE (streptavidin phycoerythrin) (Molecular Probes) and scanning the chips using a Hewlett-Packard GeneArray Scanner (Affymetrix). A second scan was performed following signal enhancement with biotinylated anti-streptavidin antibody (Vector Laboratories). The scanned images were analyzed

using the GeneChip analysis Suite 3.3 (Affymetrix) in order to identify genes differentially expressed among the RNA samples.

2.3.8 Statistical analysis

The results presented are a mean of experiments (each performed in triplicate or duplicate) ± standard error of the mean (SEM). Statistical significance was set at $p<0.05$ and was determined using ANOVA as specified in figure legends and results. P=ns is defined as "not significant".

2.4 RESULTS

ASP increases the accumulation of intracellular lipid droplets: Both 3T3-F442A and 3T3-L1 cell types were induced to differentiate with either ASP or insulin alone or both hormones together. As a measure of differentiation morphological changes were assessed by Oil-Red O staining at the end of the differentiation period. The increased accumulation of TG containing droplets was clearly visible in the cytoplasm of 3T3-F442A cells upon ASP, insulin and ASP and insulin treatment (Figure 1). Similar results were obtained for 3T3-L1cell line (results not shown).

ASP stimulates basal TG synthetic rate and TG mass accumulation during the course of differentiation: Basal TG synthetic rate in 3T3-F442A and 3T3-L1 cells was evaluated by following the incorporation of [^{3}H] oleic acid into TG during the final 4 hours of day 3, 6 and 9 of the differentiation period. As shown in Figure 2, ASP significantly increased TG synthetic rate in 3T3-F442A cells reaching 253.1±34.1% by day 9 (where basal rate at day 9 was set as 100% and was 186.0±51.6 pmols/mg cell protein/4 hours, $p<0.05$). TG mass increased concordantly and reached 231.7±35.1% by day 9 (where TG mass without treatment at day 9 was set as 100% and was 0.54±0.12 mg/mg cell protein, $p<0.001$). Similar changes were observed in 3T3-L1 cells (data not shown). The differences between treated and untreated cells were evident during the whole course of differentiation for both TG synthetic rate and TG mass. Although the effects of insulin (a known differentiation-inducing agent) were on average greater than those of ASP, the difference was not statistically significant.

Interestingly, when the 3T3-F442A cells were treated with the two hormones together (ASP and insulin), insulin significantly augmented ASP effect at day 3 ($p<0.05$) and 6 ($p<0.05$) for the basal TG synthetic rate (Figure 2, upper panel). However, for TG mass, this effect of insulin was only observed to be significantly different at day 6 ($p<0.005$) and day 9 ($p<0.005$) of differentiation period (Figure 2, lower panel). Although the ASP plus insulin effect was not significantly greater than that of insulin alone there was a trend towards additivity of ASP and insulin effects.

ASP treatment influences mRNA levels of known differentiation markers:

The expression of two transcriptional regulators believed be critical for initiation and maintenance of differentiation, C/EBP-d and PPAR-?, were examined. Cells were treated with either ASP or insulin and total RNA was isolated as described in the methods section. As shown in Figure 3, in both 3T3-F442A (Figure 3A, B) and 3T3-L1 cells (Figure 3C, D), C/EBP-d was upregulated very early in the differentiation process, reaching its maximum by day 2 and falling to a very low level by day 4 in agreement with previously published reports on expression profiles during differentiation (36). The pattern of ASP effect was comparable to insulin. On the other hand, PPAR-? gene expression persisted throughout the differentiation process with the standard insulin treatment as described previously (37), and ASP achieved the same effect as did insulin (Figure 3). The effect of simultaneous treatment of cells with ASP and insulin resulted in a similar gene expression pattern (data not shown).

The pattern of expression of genes for two enzymes involved in lipid metabolism (DGAT and adipsin) was also chosen for analysis. These genes were of particular interest because both enzymes are integral to ASP action: adipsin for generating ASP and DGAT in response to ASP stimulation. Figure 3 shows that as 3T3-F442A cells enter the differentiation program, the expression pattern of DGAT and adipsin, the late markers of differentiation, changes as well (Figure 3 A, B). Upon exposure to ASP the mRNA levels for DGAT and adipsin begin to increase from day 2 and are still on the rise at day 8 (Figure 3A). An identical pattern of induction for DGAT and adipsin was observed upon insulin

treatment (Figure 3B). Moreover, the expression level of the housekeeping gene used in our study, 18S, stays constant at all of the time points analyzed indicating that observed changes are a true representation of cellular changes. Similar results were obtained in 3T3-L1 cells (Figure 3C and 3D).

Differentiating cells undergo clonal expansion: When growth-arrested preadipocytes are induced to differentiate one of the early events that take place is mitotic clonal expansion. This phase consists of 1 to 2 rounds of cell division and happens before the cells exit the cell cycle and enter terminal differentiation. Since the first round of replication takes place in the first 24 hours, the cellular changes in DNA synthesis during that period were studied by following the incorporation of [^{3}H] thymidine into DNA. As shown in Figure 4 the increase in DNA synthesis at 24 hours after initiation of differentiation with insulin alone (3T3-F442A cells, Figure 4A) or IBMX/DX + insulin (3T3-L1 cells, Figure 4B) was evident in both cell lines, respectively. When substituted for insulin, ASP achieves similar effects. In 3T3-F442A cells (Figure 4A) ASP significantly increased [^{3}H] thymidine incorporation into DNA by 214% ($p<0.003$), insulin by 606% ($p<0.001$) whereas ASP + insulin's effect was 749% ($p<0.001$) as compared to unstimulated cells (set as 100%). In 3T3-L1 preadipocytes (Figure 4B), a similar profile was observed. In this case IBMX/DX treatment alone produced an increase in [^{3}H] thymidine incorporation into DNA by 267% above that of untreated cells ($p<0.001$, where untreated cells were set as 100%). IBMX/DX + ASP gave an additional 192% increase and IBMX/DX + insulin 252% above that of IBMX/DX alone ($p<0.001$, where IBMX/DX stimulation was set as

basal at 100%). As compared to unstimulated cells, where basal level was set at 100%, the increase was 511% with IBMX/DX + ASP and 674% with IBMX/DX + insulin (both $p<0.001$). On the other hand, 3T3-NIH cell line (a fibroblastic cell line that does not have the ability to differentiate) did not undergo clonal expansion either with insulin or ASP (Figure 4C).

cDNA microarray analysis: To further understand the effects of ASP on induction of adipocyte differentiation and to investigate possible differences from those of insulin we have analyzed the very early events of the differentiation process in 3T3-F442A cells. The gene expression profile present during the initial stages of adipogenesis was studied using cDNA microarrays containing 6500 murine genes and expressed sequence tags (EST). Microarrays were hybridized with cDNA probes from 3T3-F442A mRNA isolated at 1, 3 and 24 hours post-induction of differentiation with either ASP or insulin and induced changes in gene expression were analyzed and compared. A significant change (induction or suppression) was defined as an absolute signal difference of 300 and a fold change of 2.5. After rigorous sorting of the data from microarray analysis the results demonstrate that over the 3 time periods studied, a total of 250 genes were up regulated and 101 genes were down regulated relative to untreated cells (ASP or insulin treatment).

Figure 5 shows the levels of expression of up-regulated genes when exposed to ASP or insulin at 1, 3 and 24 hrs. When examined individually, at 3 hrs, out of the total of 53 induced genes, only 5 (9%) were induced by ASP alone, 28 (58%) by insulin alone and 20 (38%) were induced by both hormones

(Table 2). Although the level of induction varied for each gene, those that were up-regulated by both ASP and insulin were up-regulated to a similar degree (r^2=0.856, $p<0.0001$). Interestingly, while the pattern of induction was slightly different at 1 hr and 24 hrs, the proportion of up-regulated genes was on average always greater with insulin than with ASP (see Table 2). Also, by 24 hrs there were more genes induced than at 1 or 3 hrs by approximately 3 fold.

On the other hand, quite a different pattern emerged upon analysis of the genes that were suppressed when exposed to either of the two hormones. As seen in Figure 6, there tended to be more genes suppressed with ASP than with insulin alone (in contrast to induction) and little commonality was evident, especially at the early time points (1 and 3 hours, Table 3). Although, by 24 hours, genes commonly suppressed by both ASP or insulin treatment amounted to 43% of all of the suppressed genes at that time point, however, the suppression was not to a similar degree (Table 3, Figure 6). Many of these induced or suppressed genes fall in the categories of structural genes, transcription and initiation factors and we are now examining specific candidates to determine their role in ASP mediated differentiation.

2.5 DISCUSSION

To analyze the effects of ASP on the process of differentiation we have chosen to use two differentiation-competent cell lines: 3T3-L1 and 3T3-F442A. Differentiation of preadipocytes to adipocytes is obtained by addition of a simple hormonal cocktail comprising 3-isobutyl-1-methylxanthine (a cAMP phosphodiesterase inhibitor), dexamethasone (a glucocorticoid) and insulin (3T3-L1) or, simply, insulin alone (3T3-F442A). Under these hormonal conditions, changes in intracellular lipid metabolism and gene expression have been extensively studied (38,39). In this study we have demonstrated, for the first time, that by substituting ASP for insulin, both cell types can be induced to differentiate *in vitro*. While in 3T3-F442A cells ASP can be substituted directly for insulin, in 3T3-L1, addition of ASP to DX and IBMX containing media must be respected (just as seen for insulin). In order to induce adipocyte differentiation with ASP we have chosen an ASP concentration at which maximal effects on acute TG synthesis have been previously demonstrated (50 ug/ml). One possibility for the slightly smaller increases observed in basal TG synthetic rate and TG mass accumulation in the cells treated with ASP may imply that the ASP dose was not adjusted for the maximal effect on differentiation. Additional experiments exploring dose response effects of ASP on differentiation should be also performed. On the other hand, there could be a slower rate of differentiation of cells treated with ASP as compared to those treated with insulin. The additive effects of ASP and insulin may imply involvement of different signaling pathways or simply relate to a dose effect for both hormones.

The differentiation process is characterized, on one hand, by morphological changes such as cell rounding and appearance of lipid filled droplets and, on the other hand, by various changes taking place at the gene level. Ever since Green and Khinde (40) isolated differentiation-competent 3T3 clones in 1975, insulin (in addition to IBMX and DX) has been the universal hormone used to study differentiation *in vitro* and so the present knowledge of the well-ordered sequence of gene induction is defined primarily based on the insulin-induced differentiation in 3T3 cells. In this study, we observe that ASP when substituted for insulin also influences the pattern of expression of genes known as "markers of differentiation". Specifically, changes in mRNA levels of early transcription factors, C/EBP-d and nuclear receptor, PPAR-?, were observed with ASP with a profile similar to that induced by insulin. Likewise, increased expression of "late markers" of differentiation, primarily of genes necessary for lipid metabolism such as DGAT and adipsin, also followed the profile established for insulin induced differentiation (31,32). Thus, the demonstrated similarity of ASP and insulin with regards to metabolic and gene profile suggests that the observed changes are specific to the differentiation process rather than the triggering hormone.

There is a general consensus that the cascade-like recruitment of various genes is preceded by a clonal expansion phase. Immediately after hormonal stimulation, growth arrested preadipocytes re-enter mitosis and the cells undergo 1 to 2 rounds of replication (as reviewed by MacDougald (41)). It is believed that during this pre-requisite DNA synthesis phase, the unwinding of DNA results in

facilitated access of transcriptional regulators to specific regulatory regions of various genes (42). In contrast, recent data by Qiu *et al* suggest that, in 3T3-L1 cells, this step may not be necessary at all for differentiation to proceed (43), similar to observations in primary human cultured preadipocytes (44). In our study, both cell lines underwent replication within the first 24 hours from the start of induction and ASP effects paralleled those of insulin. This was specific to the induction of differentiation, since 3T3-NIH cells, which do not undergo differentiation, were not stimulated by either ASP or insulin. Interestingly, in 3T3-F442A, but not in 3T3-L1 cells, the level of stimulation, although significant, was much less pronounced for ASP than for insulin. This is perhaps the result of activation of different signaling pathways, which lead to the induction of a different set of very early genes.

Using cDNA microarray analysis, we have examined the expression of 6500 genes during the first 24 hours of differentiation. Within this very early window of initiation of differentiation, of all genes analyzed, the expression of only a few genes changed (n=351, 5% of total). The expression pattern at 24 hrs appeared to be very similar for both ASP and insulin. On the other hand, at 1 and 3 hours, ASP seems to rapidly up- and down-regulate a different set of genes than insulin. Strikingly, ASP suppresses a greater number of genes than insulin, whereas insulin induces a greater number of genes during the same time frame. It is only at the extremely early time points of 1 and 3 hours that the effects of ASP and insulin are divergent. Using clustering analysis, Friedman *et al* has shown that during *in vitro* differentiation of 3T3-L1 preadipocytes the pattern of

expression of genes can be classified into as many as 27 clusters with a number of them being suppressed in the very early hours of differentiation (45). Specific repression of various genes as a necessary regulatory step in differentiation has been noted in a number of studies. Specifically, down-regulation of the pref-1 and the Wnt gene are permissive events for the process of adipocyte differentiation (9,46).

It is not surprising to see divergence of ASP and insulin signaling immediately post-induction of differentiation since we believe that the hormones act via different receptors. Induction of differentiation by insulin is known to proceed through the IGF-1 tyrosine kinase receptor (47), whereas that of ASP is believed to involve a putative 7-transmembrane G-protein coupled receptor (12). Likewise, with regards to lipid synthesis, ASP triggers different intracellular events as compared to insulin resulting in the same biological effect of increased TG synthetic rate or TG mass. ASP acts via a protein kinase C pathway to increase fatty acid esterification (33) while insulin primarily inhibits lipolysis though its effects on cAMP phosphodiesterase and hormone sensitive lipase (48), although both also have smaller effects on lipolysis and esterification (ASP and insulin, respectively). It is not surprising, therefore, that the later phases of differentiation manifest similarities between the two hormones' actions resulting in a mature adipocyte. A recent study by Dupont at al, using cDNA microarray comparison analysis, has demonstrated that by interacting with their respective receptors, insulin and IGF-1 regulate different sets of genes, each associated with different biological effects (49). The differences in the pattern of expression

and discovery of novel genes involved in their system, is especially interesting since receptors for insulin and IGF-1 are similar in structure and activate many of the same pathways. Thus these and other studies further reinforce the notion that similarity in the hormonal or receptor structure or the final biologic outcome does not automatically predicate similarity of the signaling pathways.

Microarray analysis has now being used in a number of studies to examine the molecular events linking to the pathology of obesity. Dissection of gene profiles has been attempted by comparing preadipocyte to adipocyte 3T3-L1 gene expression during the differentiation process (50), in vivo vs. *in vitro* adipogenesis of murine cell models (45), white adipose tissue (WAT) vs. brown adipose tissue (BAT) in hamsters (51) and WAT from obese vs. diabetic mice (52). In many of these studies involvement of new and previously unknown genes is suggested. Furthermore, as demonstrated by Soukas *et al*, although the presently known transcriptional regulators (such as C/EBPs and PPAR) are believed to play an important part in the initiation and maintenance of differentiation, their data indicates the existence of additional and obligatory, but yet undiscovered, transcriptional programs (45). In many of the studies using gene chip methodology genes that are induced or suppressed group into various categories including cell division, cell adhesion, cell structure/motility, cell signaling, transcription factors, growth factors and their receptors. The majority of genes either induced or suppressed fall into category of elongation factors, transcription factors and cytoskeletal proteins. Clearly, there are still additional, as yet unappreciated, genes involved in the process of adipogenesis.

This study was undertaken to examine the potential of ASP to influence the process of adipocyte differentiation. The experimental data demonstrate clearly that at the morphological, cellular and gene levels ASP action is not limited to acute stimulation of triglyceride synthesis but, at least in 3T3 cells, may play a role in preadipocyte recruitment to adipocytes. We propose here a novel ASP function. ASP, an endogenous adipocyte factor, can increase locally in the microenvironment of the adipose tissue in response to dietary fat, and will influence not only fat accumulation in adipocytes, but also impact on neighboring preadipocytes and enhance differentiation. The consequences in terms of development of obesity remain to be seen. Interestingly, C3 (-/-) mice phenotype is characterized by reduced adipose tissue in both males and females indicating a possibility that in the absence of ASP less preadipocytes are recruited to become adipocytes. Certainly, additional studies looking at cell size vs. cell number in these knockout animals are necessary to address this question. In addition, ongoing studies are now focused on elucidation of the specific signaling pathways through which ASP mediates its effects in 3T3 cells. Identification and characterization of differentially expressed genes should help to clarify the molecular mechanisms of ASP action.

2.6 REFERENCES

1. **Rossner S.** 2002 Obesity: the disease of the twenty-first century. Int J Obes Relat Metab Disord 26:S2-S4

2. **Flegal KM, Carroll MD, Ogden CL, Johnson CL** 2002 Prevalence and trends in obesity among US adults, 1999-2000. JAMA 288:1723-1727

3. **Ludwig DS.** 2002 The glycemic index: physiological mechanisms relating to obesity, diabetes, and cardiovascular disease. JAMA 287:2414-2423

4. **Camargo CA Jr, Weiss ST, Zhang S, Willett WC, Speizer FE** 1999 Prospective study of body mass index, weight change, and risk of adult-onset asthma in women. Arch Intern Med 159:2582-2588

5. **Diamanti-Kandarakis E, Bergiele A.** 2001 The influence of obesity on hyperandrogenism and infertility in the female. Obes Rev 2:231-238

6. **Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA** 2001 The Adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. Am J Physiol Endo Metab 280:E827-E847

7. **Petruschke T, Hauner H** 1993 Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrin Met 76:742-747

8. **Greenberg AS, Nordan RP, McIntosh J, Calvo JC, Scow RO, Jablons D** 1992 Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of

mice *in vivo* and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. Cancer Res 52:4113-4116

9. **Sul HS, Smas C, Mei B, Zhou L** 2000 Function of pref-1 as an inhibitor of adipocyte differentiation. Int J Obes Relat Metab Disord 24:S15-S19

10. **Kim KH, Lee K, Moon YS, Sul HS** 2001 A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J Biol Chem 276:11252-11256

11. **Kim S, Moustaid-Moussa N** 2000 Secretory, endocrine and autocrine/paracrine function of the adipocyte. J Nutr 130:3110-3115

12. **Kalant D, Cain SA, Maslowska M, Sniderman AD, Cianflone K, Monk PN** 2003 The Chemoattractant Receptor-like Protein C5L2 Binds the C3a des-Arg77/Acylation-stimulating Protein. J Biol Chem 278:11123-11129

13. **Murray I, Sniderman AD, Cianflone K** 1999 Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. J Lipid Res 40:1671-1676

14. **Xia Z, Sniderman AD, Cianflone K.** 2002 Acylation-stimulating Protein (ASP) Deficiency Induces Obesity Resistance and Increased Energy Expenditure in *ob/ob* Mice. J Biol Chem 277:45874-45879

15. **Murray I, Sniderman AD, Havel PJ, Cianflone K** 1999 Acylation stimulating protein (ASP) deficiency alters postprandial and adipose tissue metabolism in male mice. J Biol Chem 274:36219-36225

16. **Murray I, Havel PJ, Sniderman AD, Cianflone K** 2000 Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. Endocrinology 141:1041-1049

17. **Cianflone K, Maslowska M, Sniderman AD** 1999 Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. Semin Cell Dev Biol 10:31-41

18. **Murray I, Kohl J, Cianflone K** 1999 Acylation stimulating protein (ASP): structure:function determinants of cell surface binding and triacylglycerol synthetic activity. Biochem J 342:41-48

19. **Murray I, Parker RA, Kirchgessner TG, Tran J, Zhang ZJ, Westerlund J, Cianflone K** 1997 Functional bioactive recombinant Acylation Stimulating Protein is distinct from C3a anaphylatoxin. J Lipid Res 38:2492-2501

20. **Van Harmelen V, Reynisdottir S, Cianflone K, Degerman E, Hoffstedt J, Nilsell K, Sniderman A, Arner P** 1999 Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. Journal of Biological Chemistry 274:18243-18251

21. **Cianflone K, Maslowska M** 1995 Differentiation induced production of ASP in human adipocytes. European Journal of Clinical Investigation 25:817-825

22. **Cianflone K, Roncari DAK, Maslowska M, Baldo A, Forden J, Sniderman AD** 1994 The adipsin/acylation stimulating protein system in human adipocytes: Regulation of triacylglycerol synthesis. Biochemistry 33:9489-9495

23. **Maslowska M, Sniderman AD, Germinario R, Cianflone K** 1997 ASP stimulates glucose transport in cultured human adipocytes. Intern J Obes 21:261-266

24. **Cianflone K, Xia Z, Chen LY** 2003 Critical Review of Acylation Stimulating Protein Physiology in Humans and Rodents. Biochim Biophys Acta 1609:127-143

25. **Saleh J, Summers LKM, Cianflone K, Fielding BA, Sniderman AD, Frayn KN** 1998 Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue *in vivo* in the postprandial period. J Lipid Res 39:884-891

26. **Maslowska M, Scantlebury T, Germinario R, Cianflone K** 1997 Acute *in vitro* production of ASP in differentiated adipocytes. J Lipid Res 38:21-31

27. **Scantlebury T, Maslowska M, Cianflone K** 1998 Chylomicron specific enhancement of Acylation Stimulating Protein (ASP) and precursor protein

C3 production in differentiated human adipocytes. J Biol Chem 273:20903-20909

28. **Rangwala SM, Lazar MA** 2000 Transcriptional control of adipogenesis. Annu Rev Nutr 20:535-559

29. **Student AK, Hsu RY, Lane MD** 1980 Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. J Biol Chem 255:4745-4750

30. **Fajas L, Fruchart JC, Auwerx J.** 1998 Transcriptional control of adipogenesis. Curr Opin Cell Biol 10:165-173

31. **Morrison RF, Farmer SR.** 2000 Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr 130:3116S-3121S

32. **Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Welch CB, Lusis AJ, Erickson SK, Farese RV Jr.** 1998 Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc Natl Acad Sci U S A 95:13018-13023

33. **Baldo A, Sniderman AD, St Luce S, Zhang XJ, Cianflone K** 1995 Signal transduction pathway of acylation stimulating protein: involvement of protein kinase C. J Lipid Res 36:1415-1426

34. **Neri BP, Frings CS** 1973 Improved method for determination of triglycerides in serum. Clin Biochem 19:1201-1202

35. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities or protein utilizing the principal of protein-dye binding. Analyt Biochem 72:248-254

36. **Cao Z, Umek RM, McKnight SL** 1991 Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes & Dev 5:1538.-1552

37. **Clarke SL, Robinson CE, Gimble JM.** 1997 CAAT/enhancer binding proteins directly modulate transcription from the peroxisome proliferator-activated receptor gamma 2 promoter. Biochem Biophys Res Commun 240:99-103.

38. **Gregoire FM, Smas CM, Sul HS.** 1998 Understanding adipocyte differentiation. Physiol Rev 78:783-809

39. **Cowherd RM, McGehee RE Jr.** 1999 Molecular regulation of adipocyte differentiation. Semin Cell Dev Biol 10:3-10

40. **Green H, Kehinde O.** 1975 An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19-27

41. **MacDougald OA, Lane MD** 1995 Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64:73-73

42. **Cornelius P, MacDougald OA, Lane MD** 1994 Regulation of adipocyte development. Ann Rev Nutr 14:99-129

43. **Qiu Z, Wei Y, Chen N, Wu J, Liao K** 2001 DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. J Biol Chem 276:11988-11995

44. **Entenmann G, Hauner H** 1996 Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am J Physiol 270:C1011-C1016.

45. **Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM** 2001 Distinct transcriptional profiles of adipogenesis *in vivo* and *in vitro*. J Biol Chem 276:34167-34174

46. **Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, MacDougald OA** 2000 Inhibition of adipogenesis by Wnt signaling. Science 289:950-953

47. **Smith PJ, Wise LS, Berkowitz R, Wan C, Rubin CS.** 1988 Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. J Biol Chem 263:9402-9408

48. **Elks ML, Manganiello VC** 1985 Antilipolytic action of insulin: role of cAMP phosphodiesterase activation. Endocrinology 116(5:2119-2121

49. **Dupont J, Khan J, Qu BH, Helman L, LeRoith D** 2001 Insulin and IGF-1 induce different patterns of gene expression in mouse fibroblast NIH-3T3 cells: identification by cDNA microarray analysis. Endocrinology 142:4969-4975

50. **Guo X, Liao K.** 2000 Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation. Gene 251:45-53

51. **Boeuf S, Klingenspor M, Van Hal NL, Schneider T, Keijer J, Klaus S** 2001 Differential gene expression in white and brown preadipocytes. Physiol Genomics 7:15-25

52. **Nadler ST, Stoehr JP, Schueler KL, Tanimoto G, Yandell BS, Attie AD** 2000 The expression of adipogenic genes is decreased in obesity and diabetes mellitus. Proc Natl Acad Sci U S A 97:11371-11376

Table 1: PCR primer pairs

mRNA		Primers	Gene ID #	Size	Amplification Conditions
DGAT	Sn	TTG AGC GTC TCT TAA AGC TGG	gi:3859933 AF078752	269 bp	25 cycles
	Asn	CAA AAA TAC TCC TGT CCT GGC			
Adipsin	Sn	TGT CAG AAT GCA CAG CTC C	gi:49883 XO4673	383 bp	25 cycles
	Asn	TGG TTT ACA CCC CTC AAG ACC			
C/EBP-δ	Sn	GCC TTT GAG ACT CTG AAC G	gi:6680917 NM_007679	211 bp	30 cycles
	Asn	TGT ACC TTA GCT GCA ATG G			
PPAR-γ	Sn	GCT GTT ATG GGT GAA ACT CTG	gi:18255315 BC021798.1	314 bp	30 cycles
	Asn	TGG TAT TCT TGG AGC TTC AGG			

Computer-assisted program (Primer 3; www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used to design primers. Sn=sense, Asn=antisense. Size indicates the amplified PCR fragment size in base pairs (bp). Each cycle consisted of 1 min at 95^{0}C, 1 min at 60^{0}C and 1 min at 72^{0}C.

Table 2: Analysis of genes induced in the first 24 hours post-induction of differentiation

Time	# Genes	ASP	Insulin	ASP + Insulin	Correlation	P Value
1 hr	43 (100%)	6 (14%)	11 (26%)	26 (60%)	$r^2 = 0.6178$	< 0.0001
3 hrs	53 (100%)	5 (9%)	28 (58%)	20 (38%)	$r^2 = 0.8560$	< 0.0001
24 hrs	154 (100%)	19 (12%)	32 (21%)	103 (67%)	$r^2 = 0.7778$	< 0.0001

3T3-F442A cells are induced to differentiate with either ASP (50 μg/ml), insulin (2 μg/ml) or both hormones together. The cells were collected at 1, 3 and 24 hrs post-induction of differentiation and total RNA was extracted as described in experimental procedures section. r^2 values are calculated for genes induces by both ASP and insulin. % changes are shown in brackets for each time analysed.

Table 3: Analysis of genes suppressed in the first 24 hours post-induction of differentiation

Time	# Genes	ASP	Insulin	ASP + Insulin	P Value
1 hr	11 (100%)	8 (73%)	2 (18%)	1 (9%)	ns
3 hrs	44 (100%)	32 (73%)	9 (20%)	3 (7%)	ns
24 hrs	46 (100%)	19 (41%)	7 (16%)	20 (43%)	ns

3T3-F442A cells are induced to differentiate with either ASP (50 μg/ml), insulin (2 μg/ml) or both hormones together. The cells were collected at 1, 3 and 24 hrs post-induction of differentiation and total RNA was extracted as described in experimental procedures section. r^2 values are calculated for genes induces by both ASP and insulin. % changes are shown in brackets for each time analysed.

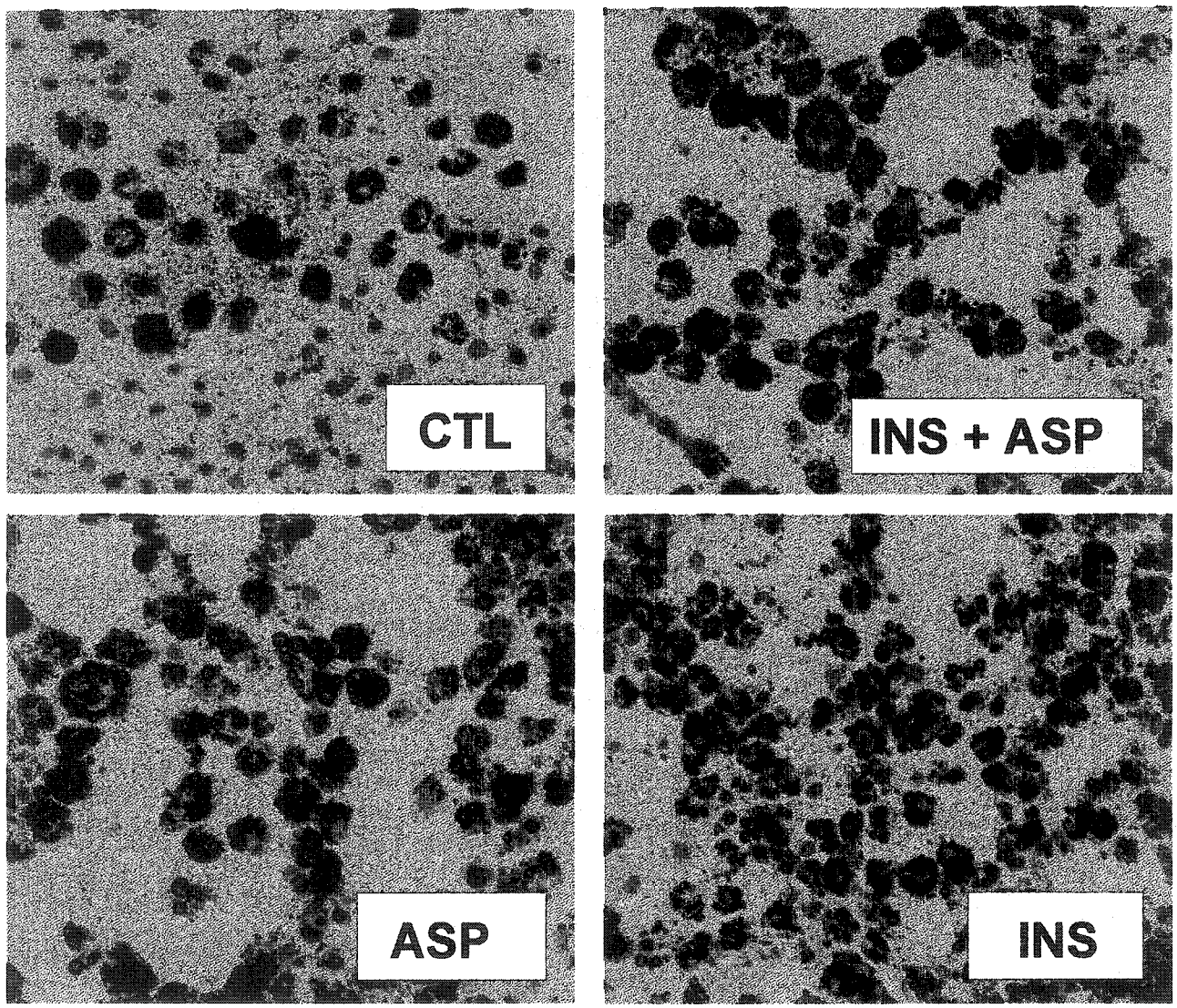
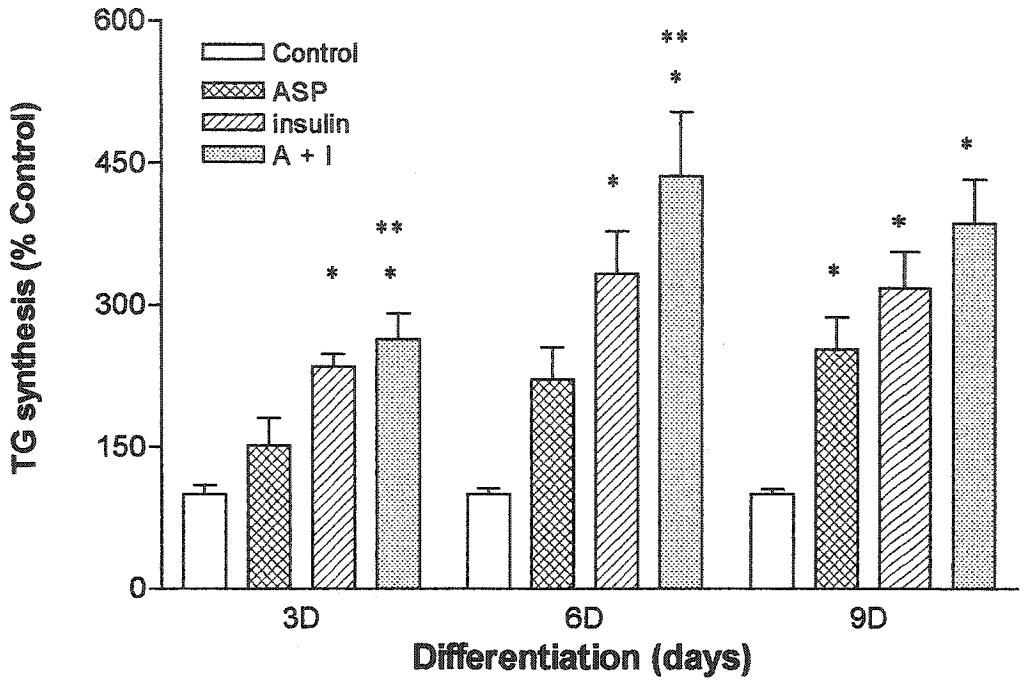

CTL
INS + ASP
ASP
INS

Figure 2.1 ASP induces morphological changes in differentiating 3T3-F442A cells. 2 days post-confluent 3T3-F442A cells were induced to differentiate with ASP (50 µg/ml) or insulin (2 µg/ml) or both hormones together over a period of 10 days. Histologic staining with Oil-Red O shows an intracellular accumulation of lipid filled droplets at day 10 of differentiation.


600
450
300
150
0
Control
ASP
insulin
A + I
TG synthesis (% Control)
3D
6D
9D
Differentiation (days)

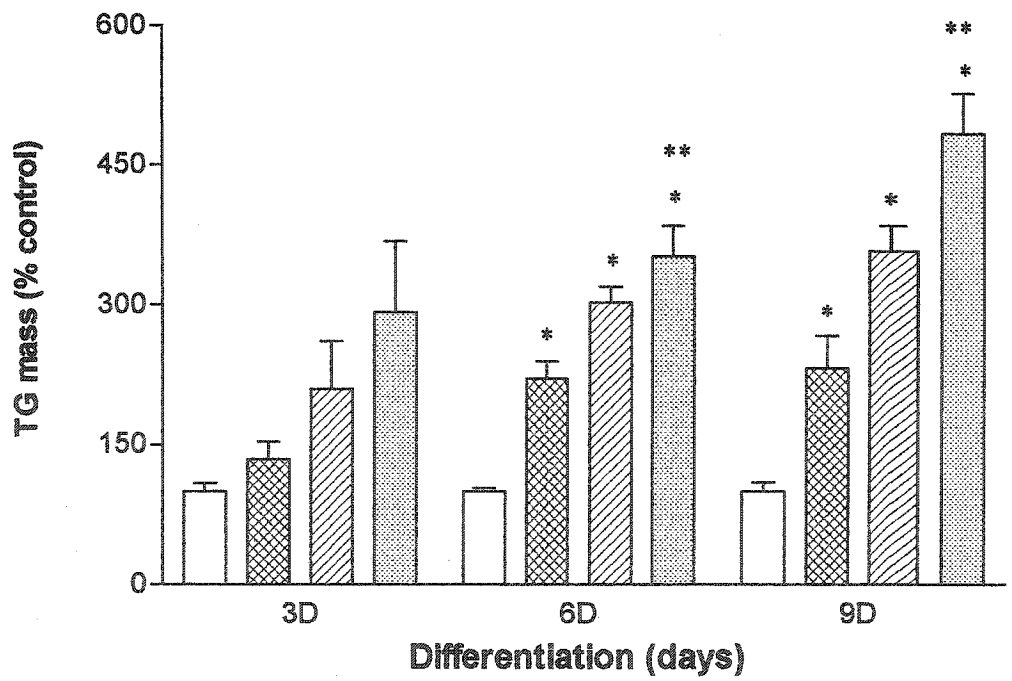

600
450
300
150
0
TG mass (% control)
3D
6D
9D
Differentiation (days)

Figure 2.2 ASP increases triglyceride synthesis and mass in differentiating 3T3-F442A cells. 3T3-F442A cells were induced to differentiate with ASP (50 µg/ml) or insulin (2 µg/ml) or both hormones together. TG synthesis (top panel) was measured as [^{3}H] oleate incorporation into triglycerides in the final 4 hour of the differentiation period (3, 6 and 9 days). TG mass (lower panel) was measured at day 3, 6 and 9 of differentiation period. Results are expressed as % stimulation ± % SEM, where basal TGS or TG mass in untreated cells was set as 100% at each time point analyzed. * $p<0.05$ (as compared to untreated cells), ** $p<0.005$ (as compared to ASP treated cells) by one-way ANOVA for n=3 experiments each performed in triplicate.

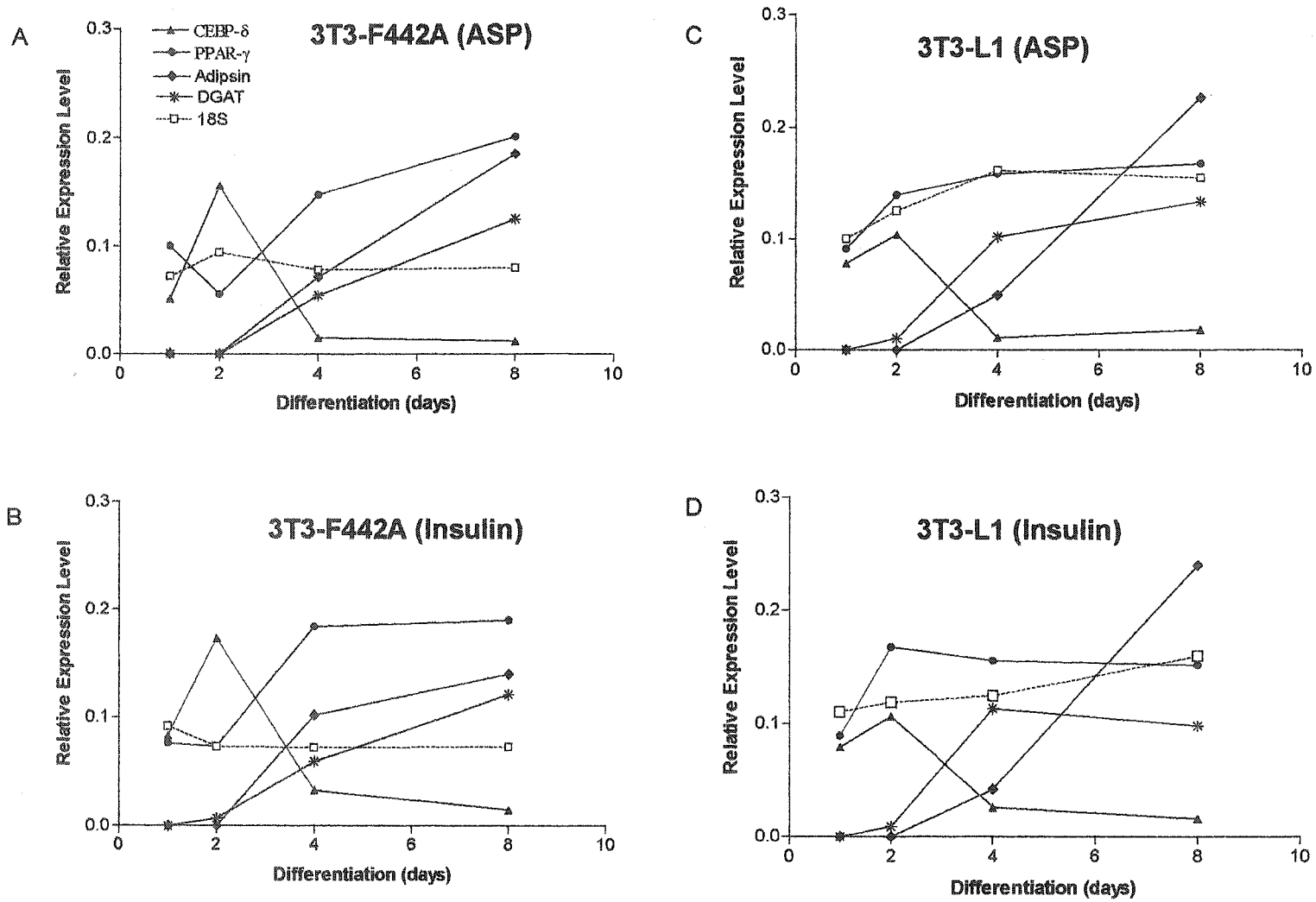

A
3T3-F442A (ASP)
CEBP-δ
PPAR-γ
Adipsin
DGAT
18S
Relative Expression Level
Differentiation (days)
B
3T3-F442A (Insulin)
Relative Expression Level
Differentiation (days)
C
3T3-L1 (ASP)
Relative Expression Level
Differentiation (days)
D
3T3-L1 (Insulin)
Relative Expression Level
Differentiation (days)

Figure 2.3 Semi-quantitative RT-PCR analysis of gene expression of C/EBP-δ and PPAR-γ (transcriptional regulators) and DGAT and adipsin (lipogenic enzymes) during differentiation. 3T3-F442A cells were induced to differentiate with ASP (A; 50 µg/ml) or insulin (B; 2 µg/ml). 3T3-L1 cells were induced to differentiate with IBMX/DX +ASP (C; 50 µg/ml) or IBMX/DX + insulin (D; 2 µg/ml). Total RNA was collected at the indicated time points following hormonal induction and the relative message levels of C/EBP-δ, PPAR-γ, DGAT, and adipsin were determined. The results are expressed as relative level of expression compared to untreated cells.

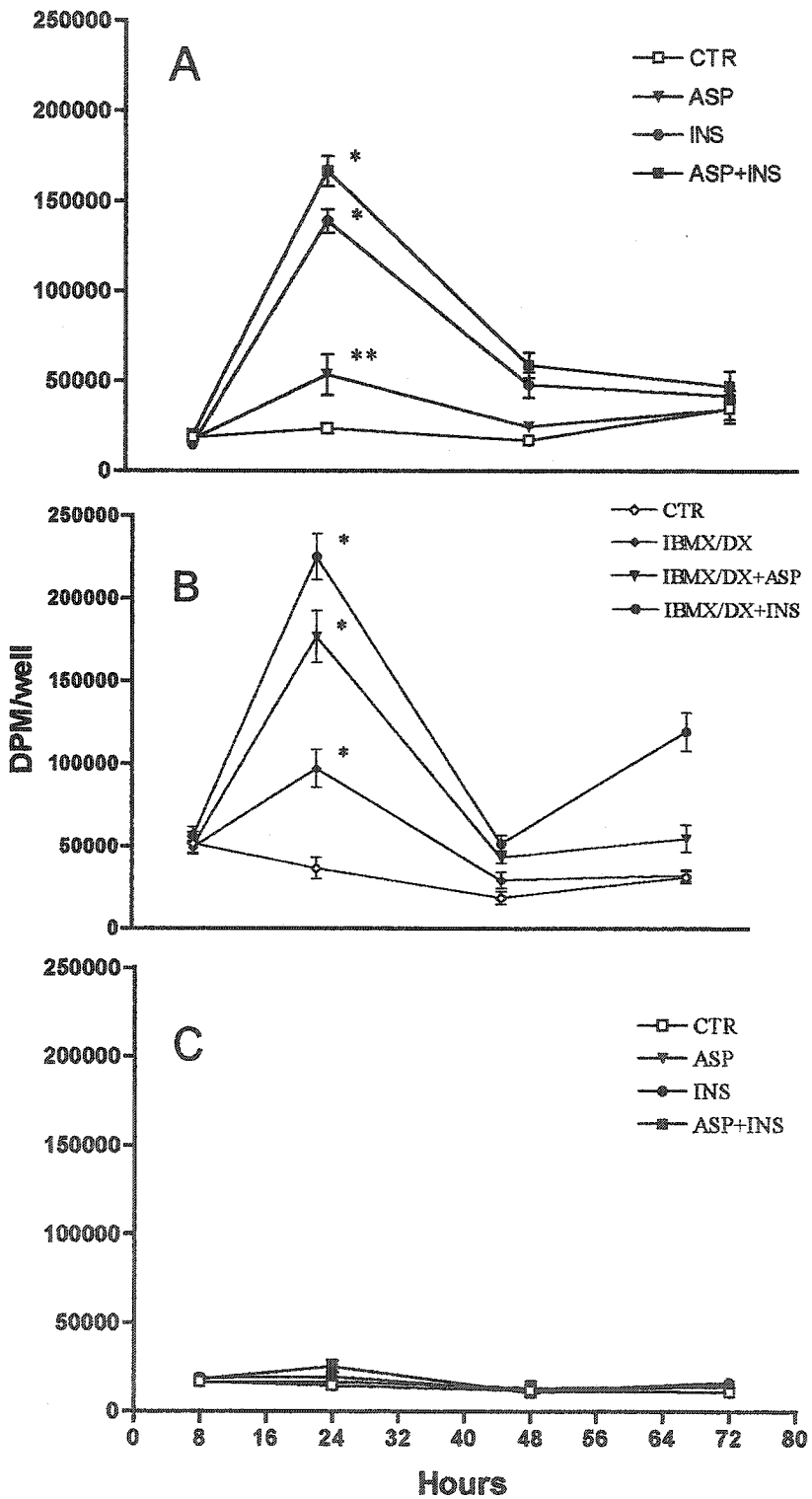
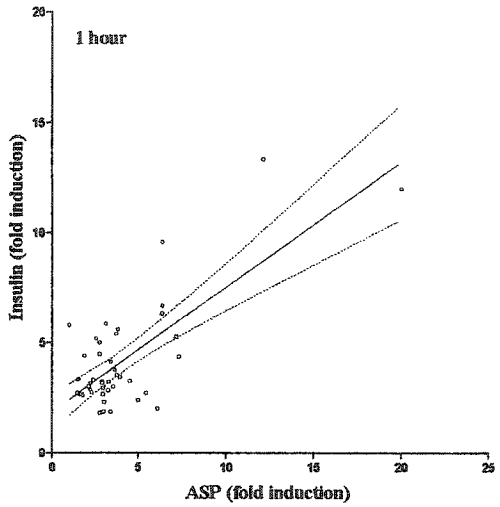
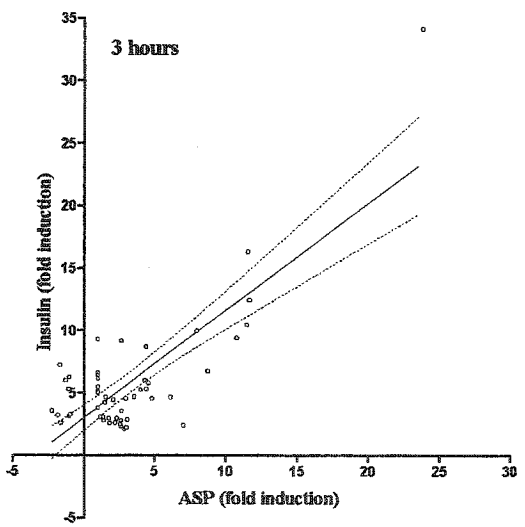

A
CTR
ASP
INS
ASP+INS
250000
200000
150000
100000
50000
0
*
*
**
B
CTR
IBMX/DX
IBMX/DX+ASP
IBMX/DX+INS
DPM/well
C
0
8
16
24
32
40
48
56
64
72
80
Hours

Figure 2.4 Mitotic clonal expansion during the induction of preadipocyte differentiation process. Post-confluent cells were hormonally induced to differentiate as described earlier. Clonal expansion was measured as [^{3}H] thymidine incorporation into DNA at the indicated time points in 3T3-F442A (A), 3T3-L1 (B) and 3T3-NIH (C) cells.


1 hour
Insulin (fold induction)
ASP (fold induction)
0
5
10
15
20
25


3 hours
Insulin (fold induction)
ASP (fold induction)
-5
5
10
15
20
25
30
35

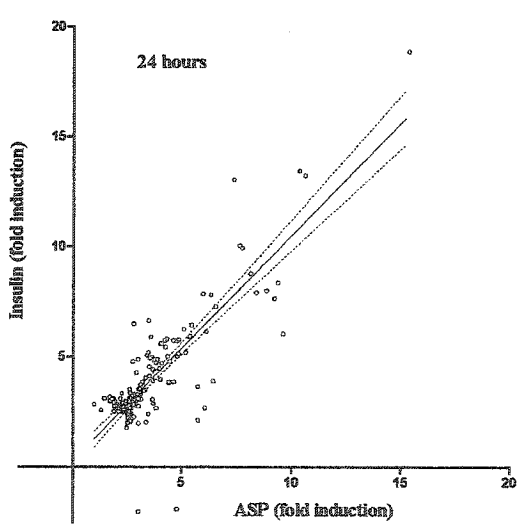

24 hours
Insulin (fold induction)
ASP (fold induction)
5
10
15
20

Figure 2.5 Scatter plot analysis of induced genes in 3T3-F442A cells. Post-confluent cells were hormonally induced to differentiate and microarray analysis was done as described earlier. For each gene, the expression level induced by ASP is plotted against that by insulin.

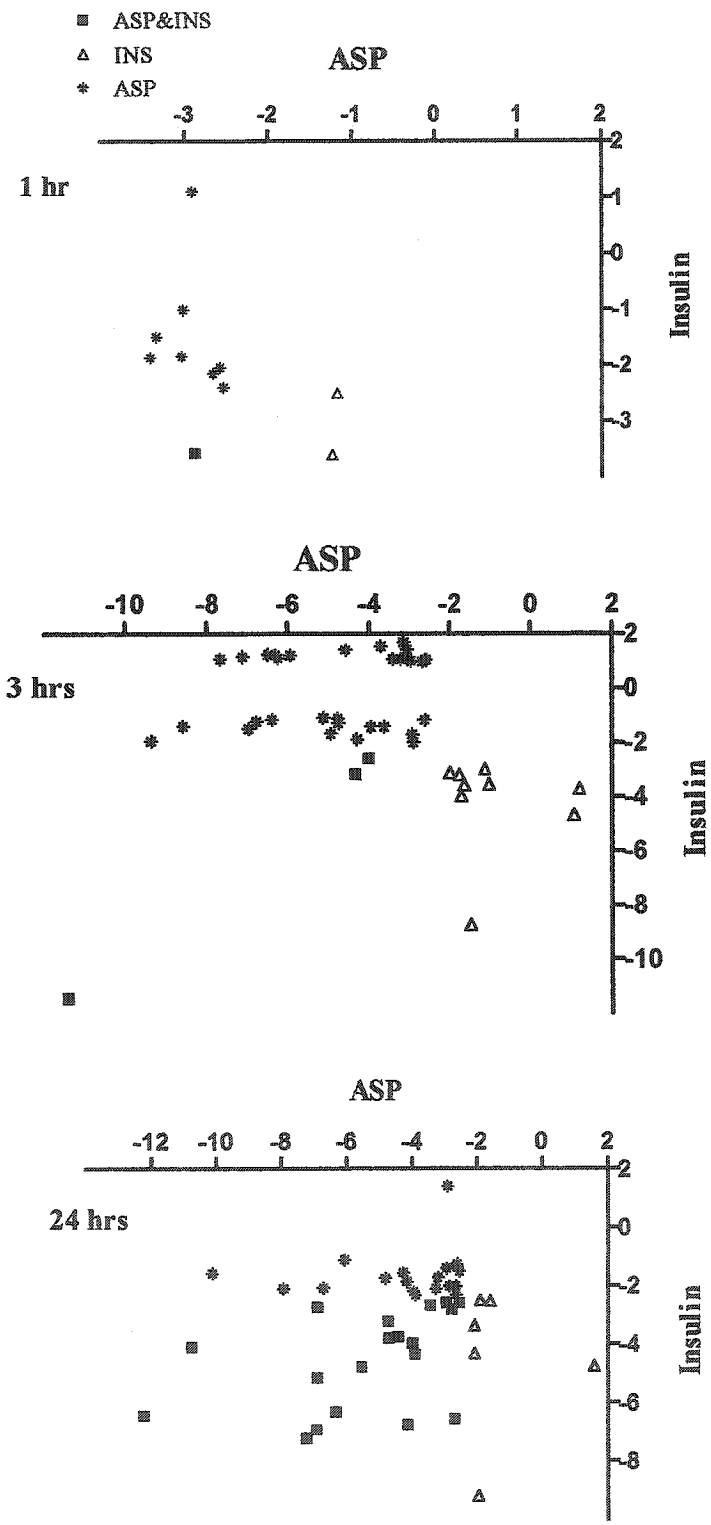

ASP&INS
INS
ASP
ASP
1 hr
-3 -2 -1 0 1 2
2 1 0 -1 -2 -3
Insulin
ASP
3 hrs
-10 -8 -6 -4 -2 0 2
2 0 -2 -4 -6 -8 -10
Insulin
ASP
24 hrs
-12 -10 -8 -6 -4 -2 0 2
2 0 -2 -4 -6 -8
Insulin

Figure 2.6 Scatter plot analysis of suppressed genes in 3T3-F442A cells. Post-confluent cells were hormonally induced to differentiate and microarray analysis was done as described earlier. For each gene, the expression level suppressed by ASP is plotted against that by insulin.

Preface II

The initiation of a biological response, such as the induction of differentiation or stimulation of TG synthesis by ASP, requires an interaction of a signaling ligand with a specific cell surface component. Although cells can receive signals in a variety of ways, in the vast majority of the cases this involves ligand binding to a specific receptor. The role of ASP in lipid metabolism has been studied in a number of ways to show that ASP has a profound effect on TG synthesis (82), glucose transport (106) and lipolysis (108). Moreover, the manuscript presented in the preceding chapter documents yet another action of ASP: an induction of adipocyte differentiation. So if ASP, similarly to insulin, is capable of all these effects, is there a receptor for ASP?

A number of studies demonstrated that ASP, in fact, interacts with a cell surface receptor. Zhang *et al* have shown that ASP binds with high affinity to human skin fibroblasts and that the binding was closely linked to its function such that decreased binding closely correlated with decreased TG synthesis and glucose transport (117). Secondly, in the study by Saleh *et al* high affinity binding of ASP in human adipose tissue was demonstrated, which was dependent on obesity status, depot site and gender (116). In addition, an important structure-function study by Murray *et al* showed that the response and binding of ASP resides in specific regions of the molecule which are different from that of the binding of its precursor, C3a, to the C3a receptor (115). However, the binding of ASP to cell surface receptor does not involve the C3a receptor. This, in turn,

could explain the observed differences in ASP vs. C3a actions in different cell types.

An orphan C5L2 receptor has recently been identified (145;146). It belongs to a subfamily of C3a, C5a and formyl Met-Leu-Phe receptors that are seven transmembrane, G protein-coupled receptors (145). Monk *et al* reported that C5L2 binds C5a, C5adesArg and C3a, although the cross-competition studies suggest that distinct sites of the receptor may be involved in the binding of these complement fragments (146). Thus, the following chapter represents experimental work characterizing ASP binding to C5L2 receptor.

Chapter 3

The Chemoattractant Receptor-like Protein, C5L2, Binds C3a desArg^{77}/Acylation Stimulating Protein

David Kalant[1*], Stuart A. Cain[2*], Magdalena Maslowska[1*], Allan D Sniderman[1], Katherine Cianflone[1] and Peter N. Monk[2]

[1]Mike Rosenbloom Laboratory for Cardiovascular Research, Division of Medicine, McGill University Health Centre, Montreal, Quebec, H3A 1A1, Canada; [2]Department of Neurology, University of Sheffield Medical School, Sheffield, S10 2RX, UK.

***These authors contributed equally to this work.**

Running title: C5L2 binds C3a desArg^{77}/ASP

Acknowledgements: This research was funded by Arthritis Research Campaign project grant M0648 (PNM) and by the Canadian Institutes of Health Research (KC).

Published in J Biol Chem 278 (13):11123-11129, 2003

3.1 ABSTRACT

The orphan receptor C5L2 has recently been described as a high affinity binding protein for complement fragments C5a and C3a that, unlike the previously described C5a receptor (CD88), couples only weakly to G_i-like G proteins (Cain, S. A., and Monk, P. N. (2002) *J Biol Chem* 277, 7165-7169). Here we demonstrate that C5L2 binds the metabolites of C4a and C3a, C4a desArg^{77} and C3a desArg^{77} (also known as acylation stimulating protein, ASP) at a site distinct from the C5a binding site. The binding of these metabolites to C5L2 does not stimulate the degranulation of transfected rat basophilic leukemia cells either through endogenous rat G proteins or when co-transfected with human $G_{\square 16}$. C3a desArg^{77}/ASP and C3a can potently stimulate triglyceride synthesis in human skin fibroblasts and 3T3-L1 preadipocytes. Here we show that both cell types, and human adipose tissue, express C5L2 mRNA and that the human fibroblasts express C5L2 protein at the cell surface. This is first demonstration of the expression of C5L2 in cells that bind and respond to C3a desArg^{77}/ASP and C3a. Thus C5L2, a promiscuous complement fragment binding protein with a high affinity site that binds C3a desArg^{77}/ASP, may mediate the acylation stimulating properties of this peptide.

3.2 INTRODUCTION

C5a and C3a have wide ranging effects in humans. Although initially described as leukocyte chemoattractants and anaphylatoxins, it is now clear that C5a and C3a are involved in microbial host defense, immune regulation (1) and protection against toxic insult (2-5). C5a and C3a are also reported to have psychopharmacological effects on feeding and drinking behavior (6,7). Both complement fragments are rapidly desarginated by serum carboxypeptidase, which modulates their function. Although C5a desArg^{74} retains most of the activity of intact C5a, albeit with a generally lower affinity for the C5a receptor ([1]CD88), C3a desArg^{77} activity is profoundly reduced relative to C3a with respect to immunologic function. No binding of the C3a desArg^{77} form to the previously cloned and characterized C3a receptor (C3aR) is observed in transfected RBL cells or mouse macrophage/monocytes (8) and, unlike C3a, C3a desArg^{77} does not stimulate eosinophil chemotaxis (9), prostanoid production by guinea pig peritoneal macrophages and rat Kupffer cells (10) or human monocyte-like U937 cell degranulation (11). However, responses to C3a desArg^{77} have been reported: cytotoxicity of NK cells is inhibited by both C3a and C3a desArg^{77} (12); cytokine production by human monocyte/macrophages and PBMC is enhanced by these ligands but inhibited in human tonsil-derived B cells (13,14) and histamine release from rat peritoneal mast cells is stimulated (15). In addition,

---

[1] CD88, human C5a receptor; C3aR, human C3a receptor; ASP, acylation stimulating protein; RBL, rat basophilic leukaemia cell line; HSF, human skin fibroblasts; PT, pertussis toxin; Bt_2-cAMP, dibutyryl cAMP; PMA, phorbol 12-myristate 13-acetate; TGS, triglyceride synthesis. FACS, fluoresence activated cell scanning.

C3a desArg^{77} has well-documented acylation stimulating properties and increases triacylglycerol synthesis in human adipocytes, preadipocytes and skin fibroblasts (HSF), where this function as acylation stimulating protein (ASP) was initially characterized (16). This triglyceride stimulating activity is also shared by C3a (17). One hypothesis explaining this pattern of responses is that cells may express two kinds of receptor: one, probably C3aR, which binds only C3a and another, as yet unidentified, receptor, which binds both C3a and C3a desArg^{77}.

We have recently characterized a novel chemoattractant binding protein, C5L2, that has high affinity for C5a, C5a desArg^{74} and C3a (18). Here we report that C5L2 also binds C3a desArg^{77}/ASP and is expressed in three C3a desArg^{77}/ASP-responsive cell types.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Cell Lines and Culture Conditions

HSF were obtained as described previously (19). RBL-2H3, HEK 293, HSF and 3T3-L1 cells were routinely cultured in Dulbecco's modified Eagle's medium or DMEM/F12 + 10% (*v/v*) fetal calf serum at 37°C, 5% CO_2. The media was supplemented with 400 mg/L G-418 for RBL-2H3 and 500 mg/L for HEK 293 stably transfected cells.

3.3.2 Stable Transfection of RBL and HEK 293 Cells

C5L2, C3aR and CD88-transfected RBL-2H3 and HEK 293 cells were produced as described (18). Ga_{16} was cloned from human monocyte mRNA and authenticated by sequencing. Human Ga_{16} and either C5L2 or CD88 were ligated into the bicistronic expression vector pIRES (Clontech). Stable transfection of RBL-2H3 cells with pIRES constructs was achieved by electroporation (20). Cells underwent three rounds of fluorescence-activated cell sorting (FACS) using anti-CD88 antibody (clone S5/1; Serotec) or anti-hemagglutinin peptide antibody (Roche Molecular Biochemicals, clone 12CA5) for C5L2 expressing cells, selecting the top 5% of receptor-positive cells in each round. HEK 293 cells were transfected (see below) then sorted with 2 rounds of FACS using FLUOS-C3a desArg^{77}/ASP binding and selecting the top 50% of the population of positive cells each time.

3.3.3 Transient Transfection of HEK 293 Cells

HEK 293 cells were seeded into 6-well plates at 1 x 10^6 cells/well the day before transfection. C5L2 in vector pEE6hCMV.neo (Celltech) or C3aR in vector pcDNA1/AMP (Invitrogen) at 2 µg DNA/well were transfected with Lipofectamine 2000 (5 µl/well) (Invitrogen) according to the manufacturer's protocol. Cells were assayed for binding/uptake three days post-transfection.

3.3.4 Production of Anaphylatoxins

Expression and purification of the recombinant His_6-tagged C5a, C5a desArg^{74} and C3a were performed under denaturing conditions as described (21). Recombinant C4a, C4a desArg^{77} and C3a desArg^{77} were expressed and purified under non-denaturing conditions by sonication in the presence of BugBuster Protein Extraction Reagent (Novagen) using manufacturer's conditions. Plasma C3a desArg^{77}/ASP and plasma C3a were purified as previously described (17).

3.3.5 Fluorescent Labelling of C3a desArg^{77}/ASP *and C3a*

C3a desArg^{77}/ASP and C3a were labeled with FLUOS (Roche Biochemicals) at a molar ratio of 1:10 (ligand to FLUOS) for two hours according to the manufacturer's recommendations. Labelled ligand was separated from free FLUOS on a Sephadex G25M column and stored in aliquots at – 80° C.

3.3.6 Radiolabelled Ligand Competition Receptor Binding Assays

Competition binding assays were performed using 50 pM ^{125}I-C5a or ^{125}I-C3a (NEN) on adherent C3aR-, CD88- or C5L2-transfected RBL cells in 96-well microtiter plates (55,000 cells/well) at 4°C as described previously (22).

Competition assays for HSF, 3T3-L1, U937 and HEK 293 were performed using 1 nM ^{125}I-C3a or ^{125}I-C3a desArg^{77}/ASP on adherent cells in 96-well microtiter plates. Competition curves were generated by pre-incubating adherent cells with increasing concentrations of unlabelled complement fragments. The IC_{50}, standard error values and linear regression analyses were obtained by using GraphPad Prism 2.0 or Sigma Plot.

3.3.7 Production of antiserum against C5L2

Antiserum was raised in rabbits using the extracellular N-terminal sequence of human C5L2 (MGNDSVSYEYGDYSDLSDRPVDC) coupled to keyhole limpet hemocyanin, as previously described (23). The serum recognized RBL cells transfected with human C5L2 (but not untransfected control cells) at dilutions as low as 1/10,000 and binding to C5L2 was totally inhibited by pre-incubation of serum with 100 µg/ml immunizing peptide.

3.3.8 Fluorescence - Activated Cell Scanning For Ligand Binding/Uptake Assays

Cells were incubated with the indicated concentrations of FLUOS-labeled C3a desArg^{77}/ASP or C3a for 30 minutes at 37°C in binding buffer (24) and washed three times with cold binding buffer. Cells were then detached with 0.25% trypsin/0.02% EDTA in PBS, fixed with 1% paraformaldehyde, washed with 0.3% phosphate-buffered saline (PBS), and assayed by fluorescence-activated cell scanning (FACS). For anti-human C5L2 binding, cells were released from the

culture dishes with non-enzymatic cell dissociation solution (Sigma chemicals), pelleted, (600g, 5 minutes), resuspended with anti-C5L2 antiserum (1: 2000 in 3% BSA in PBS) and incubated at 4^0C for 60 minutes. Again, cells were pelleted, washed twice with PBS and resuspended in FITC-labeled anti-rabbit IgG, (Sigma Chemicals, St. Louis) at (1/1000 dilution in 3% BSA in PBS) and incubated at 4^0C for 60 minutes. Finally, cells were pelleted, washed twice and resuspended in 0.3% paraformaldehyde in PBS for FACS analysis.

3.3.9 Cellular Activation Assays

Cellular activation was measured as the release of ß-hexosaminidase from RBL intracellular granules (25) or as stimulation of triglyceride synthesis in HSF and 3T3-1 cells (17). For β-hexosaminidase assays, EC_{50} and standard error values were obtained by iterative curve fitting using GraphPad Prism 2.0. For triglyceride synthesis, cells were incubated with 100 µM [^{3}H]oleate complexed to albumin (molar ratio 5:1) for 4 hours. Triglyceride synthesis was calculated as [^{3}H]oleate incorporation into triglyceride.

3.3.10 Analysis of Receptor Expression by RT-PCR

Total RNA was isolated by Trizol extraction from freshly isolated samples of the tissues and cells. For RT-PCR, cDNA was produced from 3 µg of RNA by reverse transcriptase, and 4% of the reaction was amplified by PCR with 1.5 mM $MgCl_2$ and 0.01 mM tetramethyl ammonium chloride, under the following protocol: 1 min at 94°C, 1 min at 60°C, 2 min at 72°C for 35 cycles. Primers for human C5L2 were: sense 5'-CCTGGTGGTCTACGGTTCAG-3' and antisense 5'-

GGGCAGGATTTGTGTCTGTT-3'. Primers for murine C5L2 (Ensembl gene ID: ENSMUSG00000041388) were: sense 5'-ATGGCCGACTTGCTTTGT-3' and antisense 5'-CCTTGGTCACCGCACTTTC-3'. As control, glyceraldehyde 3-phosphate dehydrogenase (GAP) was used as described previously for human GAP with human primers: sense 5'-GGTGAAGGTCGGAGTCAACGGATTTGG-3' and antisense 5'-GGCCATGAGGTCCACCACCCTGTT-3' (product size 978 bp) and mouse primers: sense 5'- CAGTTATTACCTAGTGGGG-3' and antisense 5'-CCAGTTGAGGTCTTTCCAACG-3' (product size 756 bp). Reaction products were separated on a 7.5% polyacrylamide gel and detected by silver staining (BioRad), and a 100 bp DNA ladder (NEB) was used as standard.

3.4 RESULTS AND DISCUSSION

C5L2 Is a Promiscuous Complement Fragment Binding Protein. We have previously shown that C5L2 has binding sites for C5a, C5a desArg^{74}, C4a and C3a (18). Here we show that the desArg^{77} forms of C4a and C3a are also ligands for this receptor when expressed in the RBL-2H3 cell line (Fig 1A, B, Table I) and can compete strongly with ^{125}I-C3a for C5L2 binding (Fig 1A). In contrast, C4a desArg^{77} and C3a des-Arg^{77}/ASP cannot compete effectively with ^{125}I-C5a for C5L2 or CD88 binding (Fig 1B, Table I). Although C3aR and C5L2 bind C3a with similar affinities, C3aR has no detectable affinity for C3a desArg^{77}/ASP (Table I). Similarly, although C4a can compete with ^{125}I-C3a for binding to both C3aR and C5L2, suggesting a similar affinity for both receptors, C4a desArg^{77} is more than 50-fold more effective at competing with ^{125}I-C3a binding at C5L2 than at C3aR (Table I). The data suggest either that C5L2 has two conformations with different ligand binding profiles or that the receptor has two binding sites. As we have previously shown that the B_{max} values for ^{125}I-C3a and ^{125}I-C5a binding to C5L2-transfected RBL cells are identical (18), the most likely explanation is that a single form of C5L2 has separate binding sites. We propose that one site binds ^{125}I-C3a and C3a desArg^{77}/ASP, at which all of the complement fragments except C5a desArg^{74} can compete with similar affinities and the second high affinity site, which preferentially binds ^{125}I-C5a, can only be competed by C5a desArg^{74} and, to a lesser extent, C4a.

C3a desArg^{77}/ASP binds directly to C5L2 but not to C3aR or CD88. Since recombinant C3a desArg^{77}/ASP can clearly compete with ^{125}I-C3a (but not C5a) for binding to C5L2, we then directly measured the affinity of C3a desArg^{77}/ASP for C5L2, using protein purified from human plasma as C3a desArg^{77}/ASP and tested for acylation stimulating bioactivity. Plasma purified human C3a desArg^{77}/ASP and C3a were both labeled with FLUOS. Increasing concentrations of C3a desArg^{77}/ASP were incubated with HEK 293 cells transiently transfected with C5L2, and binding and uptake assessed by flow cytometry (Fig 2A). FLUOS- C3a desArg^{77}/ASP clearly binds to C5L2, with half maximal fluorescence intensity at approximately 3 nM, whereas mock-transfected cells (Fig 2A inset) show no binding of C3a desArg^{77}/ASP even at a high concentration of 10 nM. For comparison purposes, the binding of FLUOS-C3a to HEK 293 cells transiently transfected with C3aR is shown (Fig 2B), with half-maximal binding of FLUOS-C3a at 2.5 nM. In separate experiments, FLUOS-C3a des-Arg^{77}/ASP binding to C3aR transfected cells was found to be not significantly different from basal (basal fluorescence = 100%; FLUOS-C3a desArg^{77}/ASP = 103% ±8%, mean ± S. E., n = 3) and neither FLUOS-C3a desArg^{77}/ASP nor FLUOS-C3a showed binding to cells transiently transfected with CD88, the C5a receptor (Fig 2C).

C3a desArg^{77}/ASP binding was further examined in cells that are responsive to the acylation stimulating properties of C3a desArg^{77}/ASP, and compared to HEK cells transfected with C3aR and CD88. ^{125}I-C3a desArg^{77}/ASP does not bind to C3aR-transfected HEK cells and does not compete with ^{125}I-C3a

(Table II), as found previously (26). Similarly, Bt_2-cAMP differentiated U937 macrophages (which are reported to express the C3a receptor and respond to C3a) demonstrated no specific C3a desArg^{77}/ASP binding (data not shown). The result was also negative for undifferentiated U937 cells (data not shown). As well, C3a desArg^{77}/ASP does not bind to HEK 293 cells transfected with CD88 (binding of ^{125}I-C3a desArg^{77}/ASP: mock transfection 100% ± 4% n = 6; irrelevant receptor transfection 102% ± 11%, n = 6; CD88 transfection 110% ± 22%, n = 6). Similar results were obtained for ^{125}I-C3a binding to CD88 (irrelevant receptor transfection 100% ± 6%, n = 6; CD88 transfection 99% ± 17, n = 6). By contrast, human skin fibroblasts (HSF), which respond to C3a desArg^{77}/ASP by increasing triglyceride synthesis (27), bind both ^{125}I-C3a desArg^{77}/ASP and ^{125}I-C3a with high affinity (Table II). As observed in C5L2-transfected RBL cells, unlabelled C3a desArg^{77}/ASP is slightly less effective at competing for ^{125}I-C3a binding than unlabelled C3a in both HSF and C5L2 transfected RBL cells (Tables II and I, respectively), while C3a was an effective competitor for ^{125}I-C3a desArg^{77}/ASP binding (Table II). Thus, C5L2 has binding characteristics that overlap with both CD88 and C3aR but also the unique ability to bind C3a desArg^{77}/ASP, which parallels the binding characteristics of HSF cells.

C3a desArg^{77} Binding to C5L2 Does Not Stimulate Degranulation in C5L2-Transfected RBL Cells. We have previously shown that C5a, C5a desArg^{74}, C4a and C3a binding to C5L2 does not stimulate an increase in intracellular [Ca^{2+}] nor the degranulation of transfected RBL cells, due to weak coupling to endogenous

G_i-like G proteins (18). We also examined the effects of C3a desArg^{77}/ASP and C4a desArg^{77}, and found that these ligands did not stimulate degranulation in transfected RBL cells at concentrations of up to 10 μM (data not shown). As well, there was no effect of these two ligands on either CD88 or C3aR activation of degranulation (Table III) although the expected responses to C5a, C5a desArg^{74} and C3a respectively are robust. Neither recombinant nor plasma purified C3a desArg^{77}/ASP (nor any other ligand) is able to activate endogenous G proteins in C5L2-transfected RBL cells.

Co-expression of C5L2 with $G_{\alpha 16}$ Does Not Enable a Degranulatory Response. The C5a receptor CD88 can couple effectively to the pertussis toxin (PT)-sensitive G proteins G_{i2} and G_{i3} (28) and also to toxin-insensitive G_q-family member, G_{16} (29,30). We reasoned that the moderate response of ligand coupling to C5L2 could be due to the absence of human G_{16} from RBL cells, which we tested by co-transfecting cells with human $G_{\alpha 16}$ and either CD88 or C5L2. The bicistronic vector pIRES was used, to increase the likelihood that equal amounts of receptor and G protein would be expressed in transfected cells. With transfection of CD88 alone (Fig 3A), increasing concentrations of PT inhibit the degranulation response. In co-transfected cells (Fig 3B), CD88 clearly couples strongly to $G_{\alpha 16}$, and the degranulation response to C5a is resistant to doses of PT that could substantially inhibit degranulation in cells transfected with CD88 alone. At a higher dose of PT (10 ng/ml), a small inhibition of degranulation is observed, presumably due to stabilization of interactions between free ß?

subunits and ADP-ribosylated $G\alpha_i$. In C5L2+Ga_{16} co-transfected cells, treatment with high concentrations (1 μM) of intact or desArg complement fragments still does not stimulate degranulation (Fig 3C). It appears unlikely that C5L2 couples to G proteins usually associated with leukocyte chemoattractant receptors, although this does not eliminate the possibility of coupling to other signaling pathways.

C3a desArg^{77}/ASP Stimulates Triglyceride Synthesis in Human Skin Fibroblasts But Not In Cells Expressing C3a Receptor. In HSF, both C3a desArg^{77}/ASP and C3a can stimulate triglyceride synthesis (TGS) at levels comparable to insulin, a hormone well known to influence cellular triglyceride levels (Table IV). C3a desArg^{77}/ASP appears to act via stimulation of the protein kinase C pathway (31), and stimulation of this pathway by the phorbol ester PMA also results in increased TGS (Table IV). Bioactivity of C3a is not dependent on conversion of C3a to the des-arginated form, C3a desArg^{77}/ASP, since the presence of the carboxypeptidase inhibitor (Plummer's inhibitor) has no effect on C3a bioactivity (Table IV). Increased TGS is not simply a response to C3a binding, however, as C3aR-transfected HEK cells and Bt_2-cAMP-differentiated U937 monocytic cells (which express the C3aR and bind C3a) do not respond with an increase in TGS to either C3a or C3a desArg^{77}/ASP (Table IV). However, these cell types may lack all or part of the machinery to mount an increase in TGS, as there is no significant response to treatment with PMA or insulin (Table IV).

Both C3a and C3a desArg^{77}/ASP bind to the C5L2 receptor expressed in RBL cells and HSF with comparable affinity suggesting that C5L2 may be the C3a desArg^{77}/ASP receptor on HSF. As C5L2 has already been shown to bind several complement fragments, we examined the acylation stimulating properties of other C5L2 ligands in cells that respond to C3a desArg^{77}/ASP. Even at higher concentrations than those usually used, there was no stimulation of triglyceride synthesis in 3T3-L1 preadipocytes (Table V) or in HSF (data not shown) with C5a, C5a desArg^{74}, C4a or C4a desArg^{77}, despite a clear response to C3a desArg^{77}/ASP in both cell types. Treatment of HSF or 3T3-L1 preadipocytes with other peptides of similar charge and size (lysozyme, cytochrome C) also has no effect on triglyceride synthesis or binding of C3a desArg^{77}/ASP (unpublished observations).

These results suggest that the triglyceride synthesis stimulation is both peptide and receptor specific, with both C3a desArg^{77}/ASP and C3a as the appropriate ligands interacting with the receptor C5L2. All ligands that stimulate C5L2, C3aR or CD88 to increase TGS or degranulation also act as competitors for either ^{125}I-C3a or ^{125}I-C5a binding. The converse is not true: some C5L2 ligands (e.g. C5a) bind C5L2 but fail to activate the receptor (as assessed by TGS). C4a also binds to both C3aR and C5L2 receptors but activates neither, while C3a binds to both receptors, activates both receptors but induces different responses (degranulation vs TGS). Activation requires binding to the appropriate receptor but ligand binding *per se* does not necessarily cause activation. This may be explicable in terms of the physical separation of binding and activation

sites on chemoattractant receptors such as CD88 (25,32). The two binding sites tentatively identified on C5L2 may also have different roles, one involved solely in ligand binding and one involved in both binding and activation of TGS. Thus, C5a, which binds to the first site on C5L2, may be able to sterically hinder the binding of ligands that interact primarily with the second site (C3a and C3a desArg^{77}/ASP) without activation of receptor. The ability of C5a to influence binding to the second site is presumably dependent on the C-terminal Arg residue, as C5a desArg^{74} cannot compete for ^{125}I-C3a binding to C5L2.

C5L2 mRNA and Cell Surface Protein are Expressed in Adipose Tissue, Skin Fibroblasts and 3T3-L1 Preadipocytes. Although C3a desArg^{77}/ASP is regarded as biologically inactive in most myeloid systems, the acylation stimulating properties of this complement fragment are well documented in adipocytes and related cells (33). We therefore investigated the expression of C5L2 in human adipose tissue, HSF and 3T3-L1 preadipocytes, since fibroblasts, preadipocytes and adipocytes are all known to respond directly to C3a and C3a desArg^{77}/ASP by an increase in triglyceride synthesis (Table IV) and glucose transport (17). We performed RT-PCR using species-specific sets of primers to detect expression in human adipocytes, HSF and mouse 3T3-L1 preadipocyte mRNA. Both primer sets (human and murine) produced a band as seen on polyacrylamide electrophoresis gels at sizes similar to those expected for a C5L2 transcript (Fig 4). As the DNA markers are standardized for agarose gels, not polyacrylamide gels, the human adipose tissue PCR product was extracted from an agarose gel

and sequenced. We confirmed the authenticity of the transcript as that of C5L2. By contrast, RT-PCR of RNA from the human monocytic cell line U937 and non-transfected HEK 293 cells did not result in any PCR product using C5L2 primers in spite of equal levels of glyceraldehyde-3-phosphate dehydrogenase (Fig 4).

These results were further confirmed using an antiserum specific to the N terminal region of human C5L2. FACS analysis clearly demonstrates that HSF (Fig 5A) express endogenous C5L2 on their cell surface although the fluorescent intensity was lower than that of HEK 293 cells overexpressing stably transfected C5L2 (Fig 5B). In contrast, untransfected HEK 293 cells did not bind the anti-serum (Fig 5C). As the antiserum does not appear to recognize murine C5L2 cells transfected with mouse C5L2 were negative (data not shown) and we were unable to test for expression of C5L2 on the surface of the murine 3T3-L1 cells.

In summary, we have shown that adipocytes, HSF and 3T3-L1 preadipocytes, cell types that have been shown to bind both C3a and C3a desArg^{77}/ASP and to respond to these ligands with increased triglyceride synthesis, also express C5L2. C5L2 binds both ligands with high affinity, suggesting that it may be a functional C3a desArg^{77}/ASP and C3a receptor when expressed in appropriate cell types. In contrast, C5a and C5a desArg^{74}, which bind preferentially to a different site on C5L2, do not stimulate triglyceride synthesis. The role of C5L2 in cellular responses to complement fragments is clearly complex and remains to be elucidated.

3.4 REFERENCES

1. Kohl, J. (2001) *Mol Immunol* **38**, 175-187
2. Bhatia, M., Brady, M., Shokuhi, S., Christmas, S., Neoptolemos, J. P., and Slavin, J. (2000) *J Pathol* **190**, 117-125
3. Mukherjee, P., and Pasinetti, G. M. (2001) *J Neurochem* **77**, 43-49
4. O'Barr, S. A., Caguioa, J., Gruol, D., Perkins, G., Ember, J. A., Hugli, T., and Cooper, N. R. (2001) *J Immunol* **166**, 4154-4162
5. Mastellos, D., Papadimitriou, J. C., Franchini, S., Tsonis, P. A., and Lambris, J. D. (2001) *J Immunol* **166**, 2479-2486
6. Schupf, N., Williams, C. A., Hugli, T. E., and Cox, J. (1983) *J Neuroimmunol* **5**, 305-316
7. Schupf, N., Williams, C. A., Berkman, A., Cattell, W. S., and Kerper, L. (1989) *Brain Behav Immun* **3**, 28-38
8. Zwirner, J., Werfel, T., Wilken, H. C., Theile, E., and Gotze, O. (1998) *Eur J Immunol* **28**, 1570-1577
9. Daffern, P. J., Pfeifer, P. H., Ember, J. A., and Hugli, T. E. (1995) *J Exp Med* **181**, 2119-2127
10. Puschel, G. P., Nolte, A., Schieferdecker, H. L., Rothermel, E., Gotze, O., and Jungermann, K. (1996) *Hepatology* **24**, 685-690
11. Klos, A., Bank, S., Gietz, C., Bautsch, W., Kohl, J., Burg, M., and Kretzschmar, T. (1992) *Biochemistry* **31**, 11274-11282

12. Charriaut, C., Senik, A., Kolb, J. P., Barel, M., and Frade, R. (1982) *Proc Natl Acad Sci U S A* **79**, 6003-6007

13. Fischer, W. H., Jagels, M. A., and Hugli, T. E. (1999) *J Immunol* **162**, 453-459

14. Fischer, W. H., and Hugli, T. E. (1997) *J Immunol* **159**, 4279-4286

15. Mousli, M., Hugli, T. E., Landry, Y., and Bronner, C. (1992) *J Immunol* **148**, 2456-2461

16. Cianflone, K., and Maslowska, M. (1995) *Eur J Clin Invest* **25**, 817-825

17. Murray, I., Parker, R. A., Kirchgessner, T. G., Tran, J., Zhang, Z. J., Westerlund, J., and Cianflone, K. (1997) *J Lipid Res* **38**, 2492-2501

18. Cain, S. A., and Monk, P. N. (2002) *J Biol Chem* **277**, 7165-7169

19. Cianflone, K. M., Maslowska, M. H., and Sniderman, A. D. (1990) *J Clin Invest* **85**, 722-730

20. Monk, P. N., Barker, M. D., Partridge, L. J., and Pease, J. E. (1995) *J Biol Chem* **270**, 16625-16629

21. Paczkowski, N. J., Finch, A. M., Whitmore, J. B., Short, A. J., Wong, A. K., Monk, P. N., Cain, S. A., Fairlie, D. P., and Taylor, S. M. (1999) *Br J Pharmacol* **128**, 1461-1466

22. Monk, P. N., Barker, M. D., and Partridge, L. J. (1994) *Biochim Biophys Acta* **1221**, 323-329

23. Ohno, M., Hirata, T., Enomoto, M., Araki, T., Ishimaru, H., and Takahashi, T. A. (2000) *Mol Immunol* **37**, 407-412

24. Crass, T., Raffetseder, U., Martin, U., Grove, M., Klos, A., Kohl, J., and Bautsch, W. (1996) *Eur J Immunol* **26**, 1944-1950

25. Cain, S. A., Coughlan, T., and Monk, P. N. (2001) *Biochemistry* **40**, 14047-14052

26. Wilken, H. C., Rogge, S., Gotze, O., Werfel, T., and Zwirner, J. (1999) *J Immunol Methods* **226**, 139-145

27. Cianflone, K., Vu, H., Walsh, M., Baldo, A., and Sniderman, A. (1989) *J Lipid Res* **30**, 1727-1733

28. Sheth, B., Banks, P., Burton, D. R., and Monk, P. N. (1991) *Biochem J* **275 (Pt 3)**, 809-811

29. Shum, J. K., Allen, R. A., and Wong, Y. H. (1995) *Biochem Biophys Res Commun* **208**, 223-229

30. Buhl, A. M., Eisfelder, B. J., Worthen, G. S., Johnson, G. L., and Russell, M. (1993) *FEBS Lett* **323**, 132-134

31. Baldo, A., Sniderman, A. D., St Luce, S., Zhang, X. J., and Cianflone, K. (1995) *J Lipid Res* **36**, 1415-1426

32. Cain, S. A., Woodruff, T. M., Taylor, S. M., Fairlie, D. P., Sanderson, S. D., and Monk, P. N. (2001) *Biochem Pharmacol* **61**, 1571-1579

33. Cianflone, K., Maslowska, M., and Sniderman, A. D. (1999) *Semin Cell Dev Biol* **10**, 31-41

Table I. Summary of Competition Binding Data for Human Chemoattractant Receptors Expressed in RBL Cells

	Receptor (Radioligand)							
	CD88 (^{125}I-C5a)		C3aR (^{125}I-C3a)		C5L2 (^{125}I-C5a)		C5L2 (^{125}I-C3a)	
Unlabeled Ligand	IC_{50}[1]	n[2]	IC_{50}	n	IC_{50}	n	IC_{50}	n
C5a	20.5	15	2,900	3	8.17	10	293	3
C5a desArg^{74}	411	6	9,670	3	36.9	3	>100,000	3
C4a	4,440	4	250	3	3790	4	485	4
C4a desArg^{77}	ND[3]		10,000	2	19,200	2	177	2
C3a	23,100	3	155	12	23,200	3	167	8
C3a desArg^{77}/ASP	ND		>100,000	3	18,500	3	526	3

1 IC_{50} = concentration (nM) of unlabeled ligand resulting in 50% competition of maximal radioligand binding; 2 n = number of separate experiments performed in triplicate; 3 ND = assay not done.

Table II. Competition Binding Data for Human Skin Fibroblasts and C3aR-transfected HEK Cells

	Cell Type (Radioligand)							
	Human Skin Fibroblast				HEK C3aR			
	^{125}I-C3a desArg^{77}/ASP		^{125}I-C3a		^{125}I-C3a desArg^{77}/ASP		^{125}I-C3a	
Unlabeled Ligand	IC_{50}[1]	n[2]	IC_{50}	n	IC_{50}	n	IC_{60}	n
C3a desArg^{77}/ASP	71.5±17.0	5	176±10.8*	6	No binding	3	No competition	3
C3a	50.7±4.2	5	56.6±4.2	6	ND[3]		14.0±3.7**	3

1 IC_{50} = concentration (nM) of unlabeled ligand resulting in 50% competition of maximal radio ligand binding, mean ± S.E.

2 n = number of separate experiments each performed using 15 concentrations of competing ligand each in triplicate.

3 ND = assay not done. *= significantly different from IC_{50} for C3a competition of ^{125}IC3a by paired t-test, $p<0.025$;** $p<0.001$, IC_{50} for HEK C3aR vs HSF for ^{125}I-C3a.

Table III. Summary of Receptor Activation Data for Human Chemoattractant Receptors Expressed in RBL Cells

	Receptor			
	CD88		C3aR	
Ligand	EC_{50}[1]	n[2]	EC_{50}	N
C5a	5.85	16	>10,000	2
C5a desArg[74]	21.2	5	>10,000	2
C4a	>10,000	2	>10,000	2
C4a desArg[77]	ND[3]		>10,000	2
C3a	>10,000	2	50.2	8
rC3a desArg[77] /ASP	ND		>10,000	2
Plasma C3a desArg[77]/ASP	>10,000	1	>10,000	2

1 EC_{50} = concentration of ligand (nM) resulting in 50% of maximal degranulation. 2 n = number of separate experiments performed in triplicate; 3 ND = assay not done.

Table IV. Stimulation of Triglyceride Synthesis in Different Cell Lines.

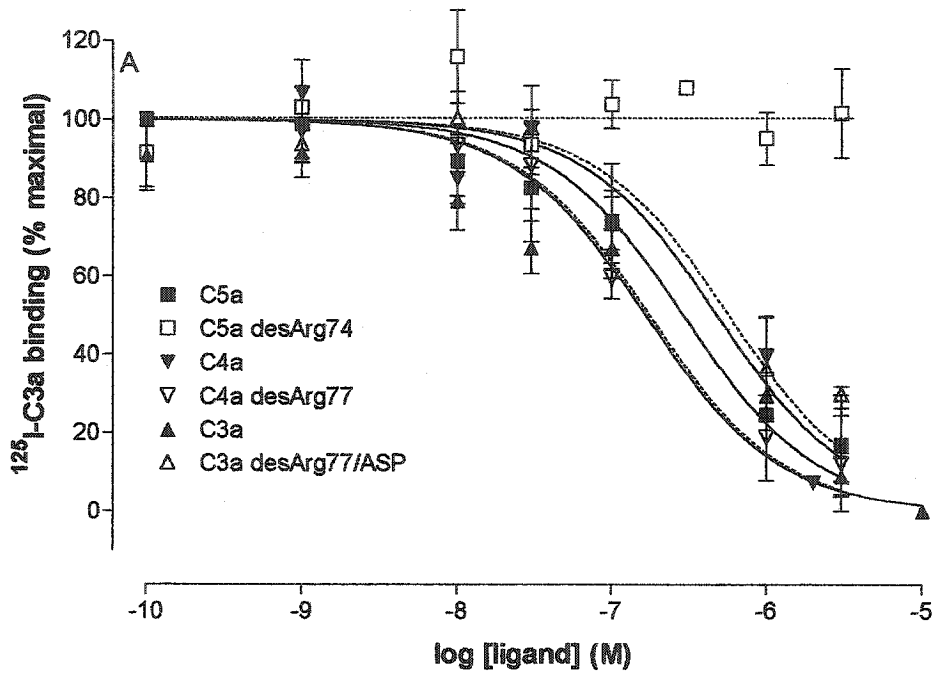
		Cell Type				
		HSF (n^1 = 5)	U937 (n=3)	Bt2cAMP-U937 (n=3)	HEK (n=3)	HEK C3aR (n=3)
Stimulus	Conc	% of Stimulation of Triglyceride Synthesis[2]				
C3a des-Arg^{77}/ASP	5μM	175.9±10.9*	115.8±32.2	115.3±25.8	111.6±8.9	95.7±7.0
C3a	5μM	212.6±27.7*	121.1±1.1	98.6±13.2	102.8±10.3	94.8±8.0
C3a + CBP[3] inhibitor	5μM	197.0±6.5*	ND[4]	ND	ND	ND
Insulin	10nM	206.4±14.3*	108.6±32.7	142.7±36.5	96.7±4.8	98.3±11.1
PMA	1nM	184.6±19.3*	70.8±35.0	103.2±17.3	136.5±12.3	88.8±12.4

1 n = number of values; 2 The results are expressed as mean ± S.E., relative to basal triglyceride synthesis (= 100%), 3 CBP = carboxypeptidase inhibitor. 4 ND = assay not done. * = significantly different from basal, $p<0.025$.

Table V. Assessment of Triglyceride Synthesis by Complement Fragments in 3T3-L1 Preadipocytes.

	Peptide				
	C3a desArg^{77} /ASP (n^{1} =12)	C5a (n=6)	C5a desArg^{74} (n=6)	C4a (n=4)	C4a desArg^{77} (n=4)
Concentration	**% Stimulation of Triglyceride Synthesis^{2}**				
1 μM	ND	91.1±6.6	100.2±4.1	106.4±2.5	105.2±3.0
2 μM	ND	101.7±13.3	100.7±3.6	90.8±5.1	104.6±7.4
5 μM	213.0±9.1*	102.6±10.1	84.9±8.5	98.4±3.5	104.6±8.0
10 μM	ND	111.2±12.6	101.2±5.7	92.6±4.6	104.3±3.7

1 n = number of values; 2 The results are expressed as mean ± S.E., relative to basal triglyceride synthesis (= 100%), * = significantly different from basal, $p<0.0001$, ND = assay not done.


A
120
100
80
60
40
20
0
^{125}I-C3a binding (% maximal)
C5a
C5a desArg74
C4a
C4a desArg77
C3a
C3a desArg77/ASP
-10
-9
-8
-7
-6
-5
log [ligand] (M)

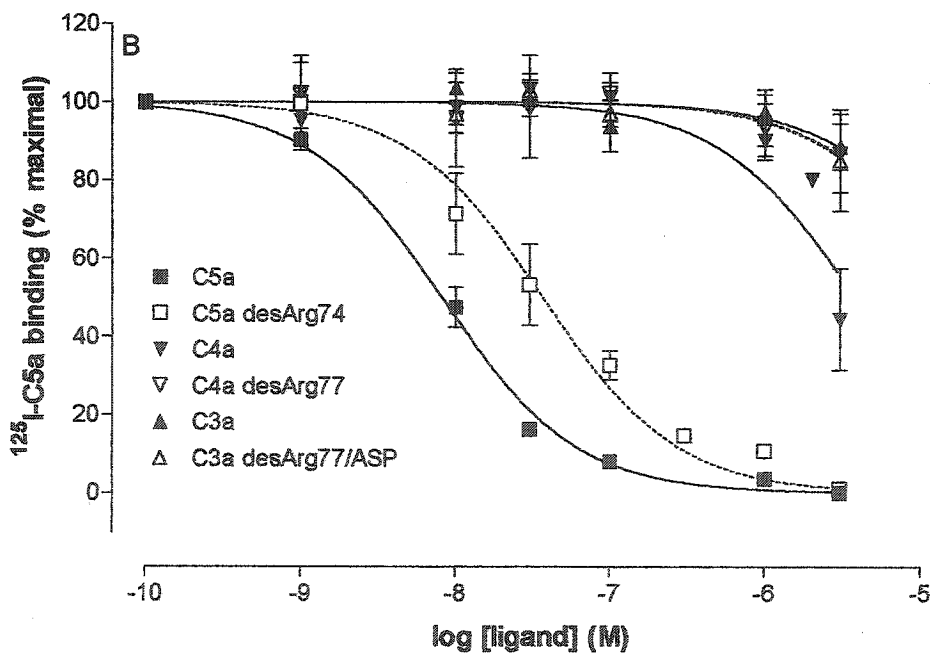

B
120
100
80
60
40
20
0
^{125}I-C5a binding (% maximal)
C5a
C5a desArg74
C4a
C4a desArg77
C3a
C3a desArg77/ASP
-10
-9
-8
-7
-6
-5
log [ligand] (M)

Figure 3.1 C3a desArg^{77}/ASP and C4a desArg^{77} Bind to RBL Cells Expressing C5L2. RBL cells stably transfected with C5L2 were incubated with the stated concentrations of complement fragments for 10 min prior to the addition of 50 pM ^{125}I-C3a (A) or 50 pM ^{125}I-C5a (B). Results are the means of n (n shown in Table 1) separate experiments performed in triplicate ± S.E.

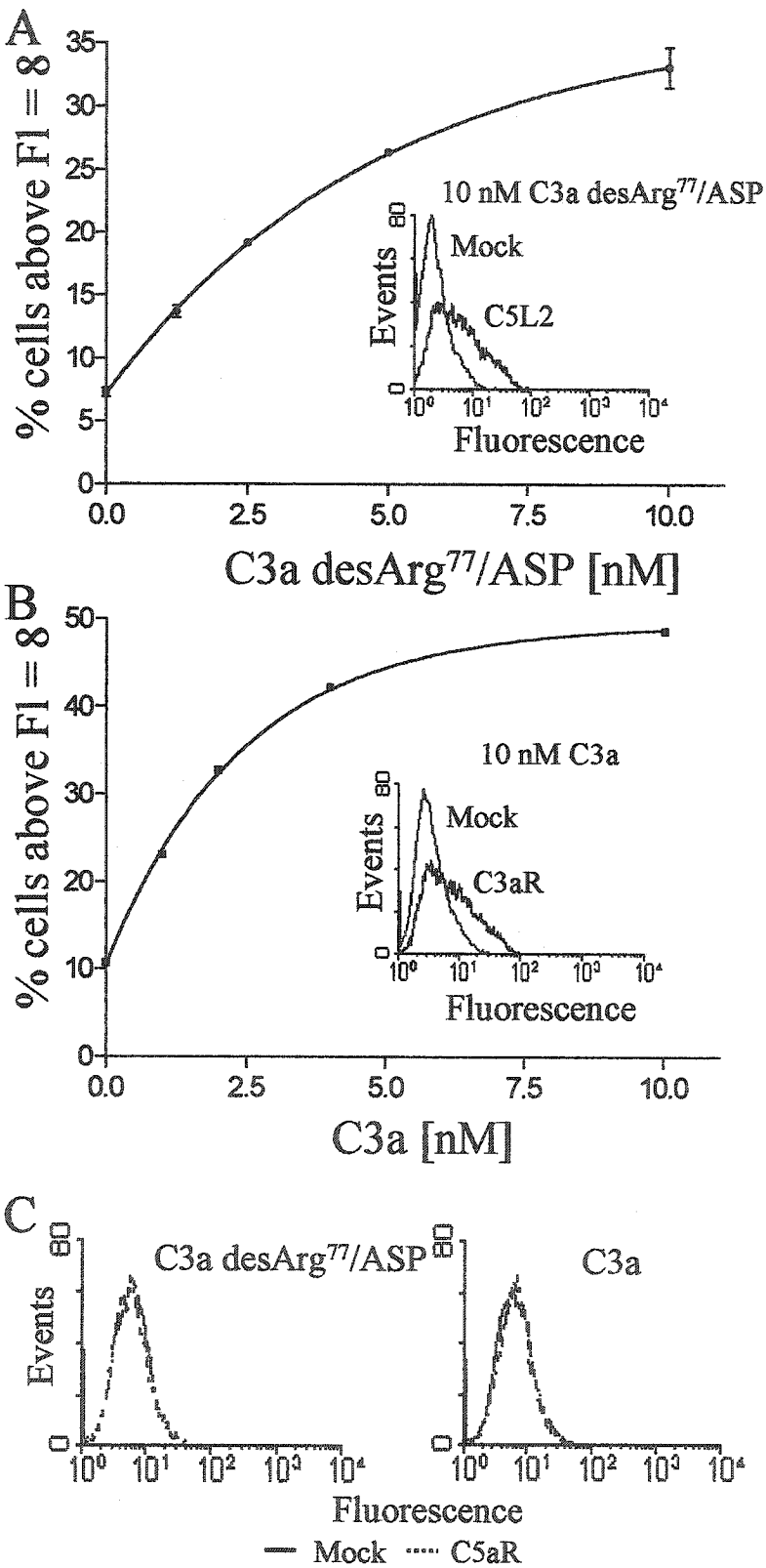
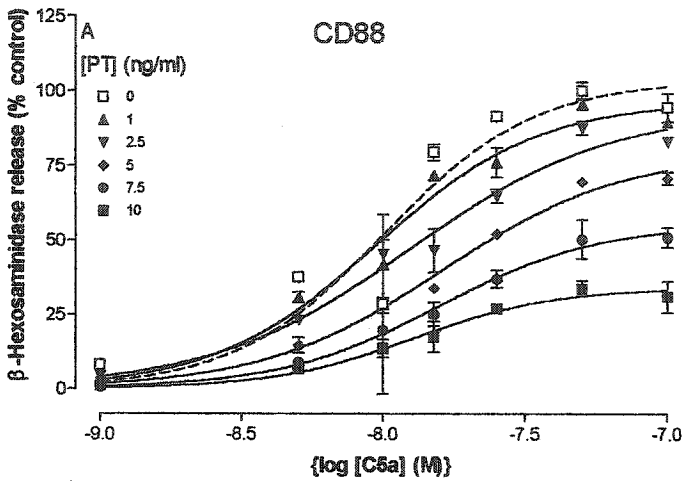
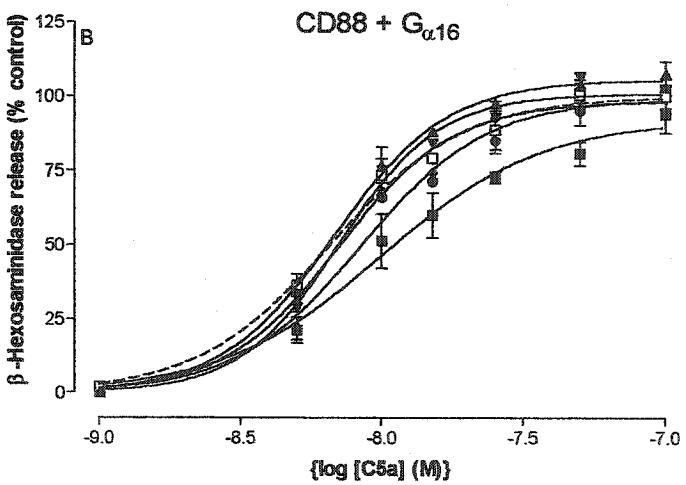

A
% cells above F1 = 8
35
30
25
20
15
10
5
0
0.0
2.5
5.0
7.5
10.0
C3a desArg77/ASP [nM]
10 nM C3a desArg77/ASP
Events
80
0
Mock
C5L2
10^0 10^1 10^2 10^3 10^4
Fluorescence
B
% cells above F1 = 8
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
C3a [nM]
10 nM C3a
Events
80
0
Mock
C3aR
10^0 10^1 10^2 10^3 10^4
Fluorescence
C
Events
80
0
C3a desArg77/ASP
C3a
10^0 10^1 10^2 10^3 10^4
Fluorescence
— Mock ····· C5aR

Figure 3.2 C5L2 shows Saturable Binding/Uptake of C3a desArg^{77}/ASP. HEK 293 cells were transiently transfected with C5L2 (A), C3aR (B) or CD88 (C) and three days later cells were incubated for 30 min with the indicated concentrations of FLUOS-labeled C3a desArg^{77}/ASP or C3a, respectively. Binding/uptake was assessed by FACS, and the percentage of cells above a fluorescence intensity of 8 was determined. The fluorescence histograms for mock vs receptor transfected cells at the highest ligand concentration are shown in the insets (A and B).


A
CD88
[PT] (ng/ml)
0
1
2.5
5
7.5
10
β-Hexosaminidase release (% control)
125
100
75
50
25
0
-9.0
-8.5
-8.0
-7.5
-7.0
{log [C5a] (M)}


B
CD88 + Gα16
β-Hexosaminidase release (% control)
125
100
75
50
25
0
-9.0
-8.5
-8.0
-7.5
-7.0
{log [C5a] (M)}

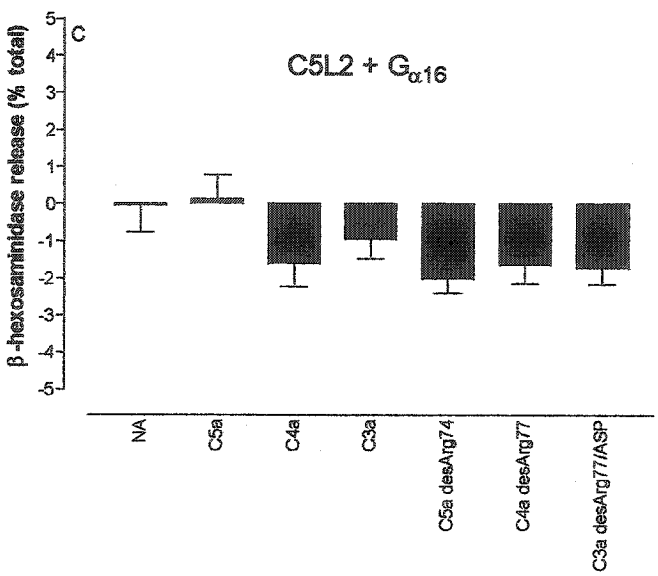

C
C5L2 + Gα16
β-hexosaminidase release (% total)
5
4
3
2
1
0
-1
-2
-3
-4
-5
NA
C5a
C4a
C3a
C5a desArg74
C4a desArg77
C3a desArg77/ASP

Figure 3.3 CD88 but not C5L2 Stimulates Degranulation Coupled to $G_{\alpha 16}$ Proteins. RBL cells were transfected with human CD88 (A), CD88+$G_{\alpha 16}$ (B), or C5L2+$G_{\alpha 16}$ (C) using monocistronic or bicistronic expression vectors. Functional association of CD88 with $G_{\alpha 16}$ was demonstrated using pertussis toxin treatment to inhibit endogenous G_i-like G proteins: CD88 (A) and CD88+$G_{\alpha 16}$ (B) transfected RBL cells were treated for 4 h with 0 – 10 ng/ml pertussis toxin (PT) prior to the addition of C5a. Degranulation was measured as secretion of ß-hexosaminidase, expressed as a percentage of the maximal release in the presence of 1 µM C5a with no PT. Typical release under these conditions was 80% of total cellular ß-hexosaminidase. (C) RBL cells transfected with C5L2+$G_{\alpha 16}$ were treated with 1 µM of the indicated complement fragments and degranulation measured as secretion of ß-hexosaminidase, expressed as a percentage of the total cellular content.

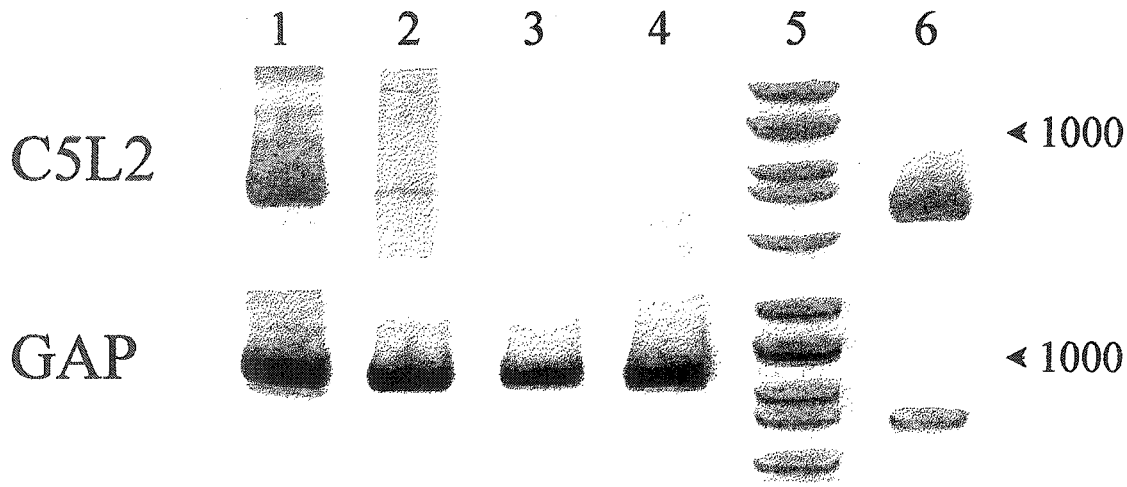

1
2
3
4
5
6
C5L2
GAP
1000
1000

Figure 3.4 C5L2 is Expressed in Cells That Show Binding and Response to C3a desArg^{77}/ASP and C3a. RT-PCR of human adipose tissue, human skin fibroblasts and mouse 3T3-L1 preadipocytes with primers for C5L2 show bands of expected size (human - 798 bp, mouse - 739 bp) after polyacrylamide gel electrophoresis and silver staining. Cell lines that are negative for C3a desArg^{77}/ASP binding and response (HEK 293, U937 monocytic cells) show no band. For control, human (Lanes 1-4) and murine (Lane 6) glyceraldehyde 3-phosphate dehydrogenase (GAP) was used. Lane 1; human adipose tissue. Lane 2; human skin fibroblasts. Lane 3; HEK 293. Lane 4; U937 monocytic cells. Lane 5; 100 bp DNA ladder with 1000 bp indicated. Lane 6; 3T3-L1 preadipocytes.

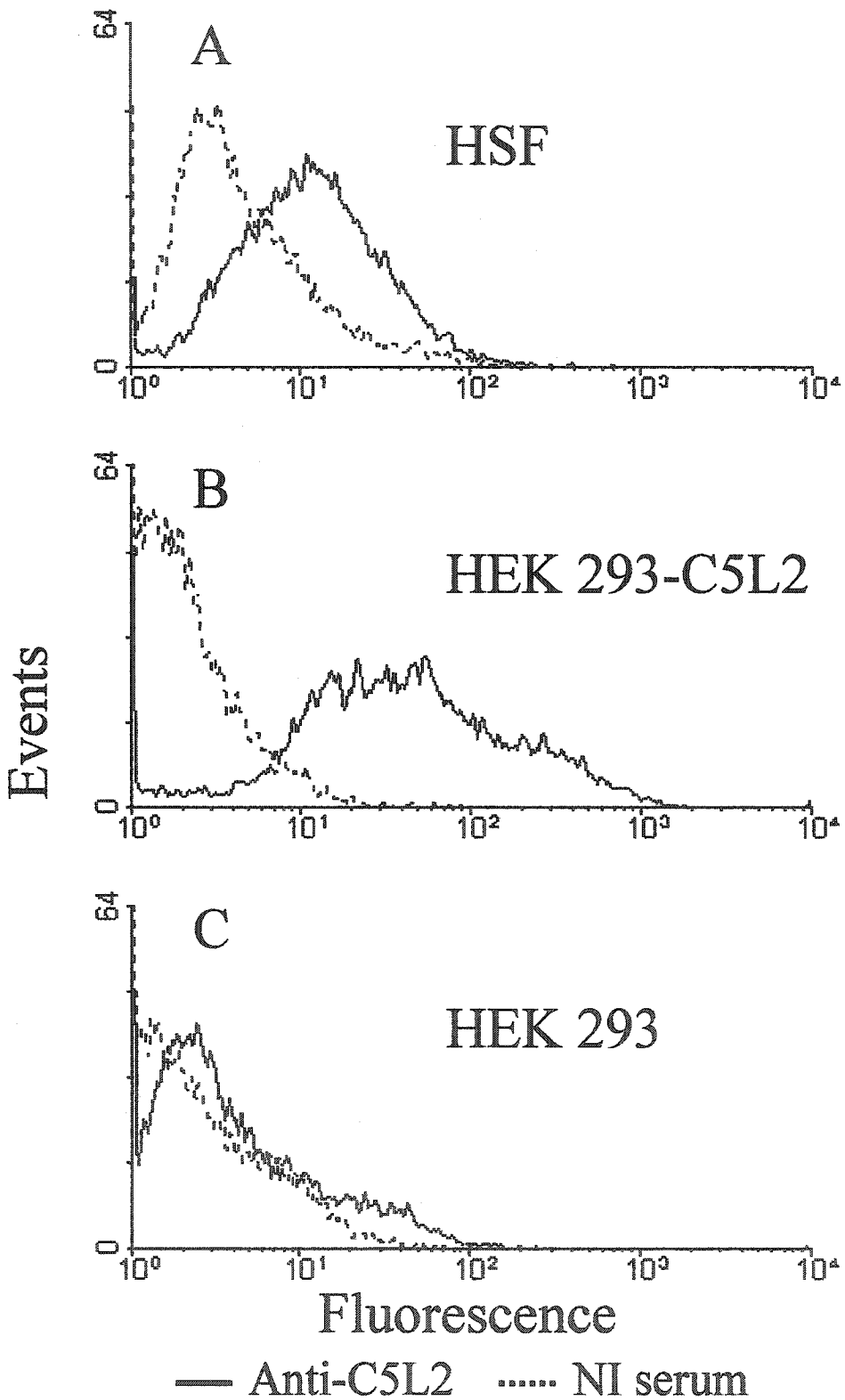

A
HSF
64
0
10^0
10^1
10^2
10^3
10^4
B
HEK 293-C5L2
C
HEK 293
Events
Fluorescence
— Anti-C5L2 ..... NI serum

Figure 3.5 Human Fibroblasts Demonstrate Cell Surface Expression of Human C5L2. HSF cells (A), HEK 293 cells stably transfected with C5L2 (B), and untransfected HEK 293 cells (C) were detached nonenzymatically and incubated at 4^0 C with either rabbit anti-C5L2 (intact line) or rabbit non-immune serum (NI; broken line) as control. After washing, the cells were incubated with goat anti-rabbit IgG conjugated to FITC. After washing and fixing with paraformaldehyde, cellular fluorescence was measured by FACS.

Preface III

The potential of an organism or an individual cell to respond to the incoming signal is crucial to its survival. There is little doubt that ASP actions invoke a variety of effects, all relating to the acute regulation of lipid metabolism. In chapter 2, we have shown yet another effect for ASP, the potential to regulate the differentiation of preadipocytes. In chapter 3, we identified the first ASP receptor and demonstrated its presence on cells responsive to ASP. In the following chapter we address the issue of intracellular signaling of ASP, an important aspect, linking interaction of ASP with receptor to the ASP effects.

Despite published data on the physiological role of ASP, little is known of the intracellular signaling pathway. The first signal transduction studies on ASP demonstrated activation of protein kinase C (PKC) but not protein kinase A (PKA) enzyme (109). This was complemented by the observation of transient increases in diacylglycerol, an intracellular lipid signaling molecule responsible for PKC activation. However, the final event in most of the cell signaling pathways is the modification of the activity of an enzyme or an activation factor. We have experimental evidence suggesting that stimulation of TG synthesis by ASP involves activation of diglyceride acyltransferase (DGAT) (104), the final and specific enzyme responsible for the formation of triglyceride. While the results presented in Chapter 4, using various inhibitors, shed light on the specific intracellular players involved in the ASP-stimulated TGS, they are still preliminary. They also indicate that basal TGS may be controlled by different intracellular metabolic pathway.

Chapter 4

Targeting the signaling pathway of Acylation Stimulating Protein

Work in progress 2003

4.1 INTRODUCTION

Adipose tissue stores energy in the form of fat. The surplus energy that is assimilated by the fat cells is stored as triglyceride in lipid droplets. Augmentation of adipose tissue leads to obesity which, in turn, leads to a number of metabolic perturbations such as diabetes, coronary artery disease (CAD) and hypertension. Presently, obesity is one of the most common health problems in our society (147). It is, also, one of our biggest challenges in understanding how to control its development.

Adipose tissue, in addition to being a storage organ, produces a variety of adipokines some of which are involved in regulation of lipid metabolism in an autocrine manner (for review see (148)). Acylation Stimulating Protein (ASP) is produced by the adipose tissue through the interaction of Factor B and adipsin with complement C3. We have shown that ASP production along with Factor B, adipsin and C3 is increased during the process of differentiation of human preadipocytes, an *ex vivo* cell model (89). This production can further be augmented by the chylomicron lipoprotein plasma component (dietary fat) (96). *In vivo*, production of ASP by adipose tissue has been shown by Saleh *et al* (95). By studying arterial-venous differences in microcirrculation of adipose tissue, they have shown that in the postprandial period ASP production by the adipose tissue increased and correlated strongly with plasma triglyceride (TG) clearance. In obesity, the levels of ASP are increased (91) as they are in CAD patients (92;93).

ASP stimulates esterification of fatty acids into intracellular triglyceride in human skin fibroblasts, human preadipocytes and adipocytes (102;103). The effects of ASP on differentiated human adipocytes are much greater than those observed in preadipocytes (82). In stimulating TG synthesis, ASP has been shown to increase activity of diacylglycerol acyltransferase (DGAT, the enzyme that mediates the final step the TG synthetic pathway) in the microsomal preparations from adipocytes (104). In addition, ASP effects on glucose transport (due to translocation of glucose transporters) have been demonstrated in a variety of cell systems (105-107). However, the contribution of ASP-stimulated glucose transport to TG synthesis is relatively minor. How the effects of ASP are signaled from the surface receptor to the inside of the cell resulting in increased TG synthesis is presently not yet understood nor are the main components of the TG signaling cascades known in detail.

ASP stimulatory effects involve activation of PKC and biphasic modulation of diacylglycerol levels in the first hour post ASP treatment (109). Based on this information, the aim of the present study was to determine which other intracellular enzymes are participating in the activation of TG synthesis by ASP. Although initially murine 3T3-L1 preadipocytes cell model will be used, later on the signaling pathway will also be evaluated in 3T3 adipocytes providing an elegant way to compare ASP signaling pathway between preadipocytes and adipocytes. 3T3 preadipocytes will be exposed to various compounds specific for inhibition of the major known intracellular pathways and TG synthesis will be stimulated with ASP. U731222, n-butanol and isotetrandrine were used to study

involvement of phospholipases (PLC, PLD and PLA_2, respectively). Employing wortmannin and LY294002 addressed the involvement of phosphatidylinositol 3-kinase (PI3K). Rapamycin and PD098059 were used to establish the participation of p70 ribosomal protein-S6 kinase (p70 S6 kinase) and mitogen-activated protein kinase pathway / extracellular-signal-regulated kinases (MAPK/ERK1/2), respectively.

4.2 MATERIALS AND METHODS

4.2.1 Materials

3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA, USA). All tissue culture reagents such as Dulbecco's minimum essential medium (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS) and trypsin were from Gibco (Burlington, On, Canada). Inhibitors used were from CalBiochem (pertussis toxin, isotetrandrine, PD098059, wortmannin, rapamycin; LaJolla, CA, USA), Promega (LY294002; Madison, Ws, USA), Sigma (U73122; Oakville, On, Canada). Stimulators of TG synthesis, insulin and phorbol 13-myristate 12-acetate (PMA), were from Sigma. Oleic acid [9,10-^{3}H(N)] was from DuPont-New England Nuclear (Mississauga, On, Canada). Thin-layer chromatography plates (silica gel 150A) came from Fisher (Nepean, On, Canada). Organic solvents, scintillation vials, general chemicals and tissue culture materials were from VWR (Montreal, QC, Canada). Scintillation fluid was from ICN (Costa Mesa, CA, USA). BioRad reagent for protein measurements was from BioRad (Mississauga, On, Canada).

4.2.2 ASP preparation

ASP was isolated and purified from human plasma as described previously (82). Each batch was verified for purity by ion-spray mass spectrometry at McGill University Mass Spectrometry Unit. The activity of each ASP preparation was checked by its ability to stimulate triglyceride synthesis in 3T3-L1 preadipocytes.

4.2.3 Cell culture and experimental conditions

3T3-L1 preadipocytes, maintained at low passage number, were grown in DMEM/F12 media supplemented with 10% FBS. At 80% confluency, the cells were plated for experiments at 7,000 cells/ well on 24 well plates. The experiments were performed on the 4^{th} day after plating at 100% cell confluency. The preadipocytes were placed in serum-free medium (DMEM/F12) for 2 hours prior to initiation of the experimental treatment. To initiate the experiments the cells were first preincubated in serum-free media containing various inhibitors for 30 minutes (isotetrandrine, n-butanol, U73122, wortmannin, LY294002, rapamycin, PD98059) or 4 hours (pertussis toxin). Subsequently, the media was changed to fresh serum-free DMEM/F12 supplemented with 100 μM [^{3}H] oleate:BSA (1:5) (average specific activity 65 dpm/pmol). Inhibitors, which were reconstituted and stored according to the manufacturer's instructions, were added at appropriate concentrations from freshly prepared working solutions diluted in PBS. Finally, ASP (5 μM) and insulin (100 nM) were added as well and triglyceride synthesis was measured over 4 hours at 37°C and 5% CO_2. Appropriate vehicle controls were performed in each experiment.

4.2.4 Intracellular triglyceride synthesis assay

After the incubation period radioactive media was discarded and the cells were washed two times in ice-cold PBS. The lipids were extracted overnight in 1 ml of heptane : isopropanol (3:2 *v/v*) then rinsed with an additional 1 ml of the same

solvent mix. Lipid extracts were evaporated to dryness in the centrifuge-evaporator (Canberra-Packard, Canada), redissolved in 100 ul of chloroform : methanol (2:1 *v/v*) and were resolved by thin-layer chromatography (TLC). TLC plates were run in hexane : ethyl ether : acetic acid (75:25:1 *v/v*) with reference lipids run concurrently. The resolved lipids were visualized with iodine vapor and the spots corresponding to triglyceride were scraped into scintillation vials and counted by liquid scintillation counting (Beckman, CA). Cell proteins were solubilized in 0.1 N NaOH for 3 hours and measured by the method of Bradford (149).

4.2.5 Statistical analysis

The results were normalized (where basal TG synthesis without additions was set as 100%) for each experiment and are presented as mean ± standard error of the mean. Differences between means were analyzed using Student's *t*-test, with $P<0.05$ considered to be significant and n represents number of experiments each performed in duplicate or triplicate.

4.3 RESULTS AND DISCUSSION

The ASP receptor, C5L2, belongs to a large family of seven - transmembrane, G protein-coupled receptors (145). In this scenario, ligand binding to the receptor will result in receptor activation, which in turn will activate a Gα subunit (through GDP to GTP exchange) of the trimeric complex made of Gα, Gβ and Gγ, causing its dissociation from the receptor itself and from its partners - β/γ. There are 4 major Gα families ($G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$) each comprising a number of different isoforms and each responsible for activating a different set of intracellular enzymes and pathways (150). Activation of the receptor coupled to the $G\alpha_s$, activates adenylyl cyclase and results in release of cAMP, which in turn, activates protein kinase A (PKA) (151). We have previously demonstrated, by using cholera toxin ($G\alpha_s$ stimulator), that $G\alpha_s$ was not involved nor was PKA activated in ASP signaling mechanism (109).

In the present study, pertussis toxin was used to evaluate the involvement of $G\alpha_i$ subunit. $G\alpha_i$ subunit is analogous to $G\alpha_s$, except on activation and diffusion to adenylyl cyclase the release of cAMP is depressed. Pertussis toxin modifies the C-terminal portion of $G\alpha_i$ and in so doing prevents its interaction with the receptor and its activation. The lack of activation of the $G\alpha_i$ protein stops the inhibition of adenylyl cyclase (152). Confluent 3T3-L1 preadipocytes were treated for 4 hours with 100 ng/ml pertussis toxin prior to the addition of 5 μM ASP (a standard, maximum stimulatory dose for ASP on TGS). At this concentration, ASP has been shown to be maximal for TG synthetic activity in 3T3-L1 preadipocytes. The cells were then incubated for an additional 4 hours. The

results are shown in Figure 1. The magnitude of TGS stimulation with ASP in the absence (181.9±14.6%) *vs.* presence (216.3±10.7%) of 100 ng/ml pertussis toxin was comparable indicating that pertussis toxin does not inhibit the ASP effect (P=NS). Insulin also stimulates TGS in 3T3-L1 preadipocytes. These effects are believed to be a result of insulin binding to the insulin receptor, which belongs to the family of tyrosine kinase receptors. As expected, pertussis toxin had no inhibitory effect on the TGS stimulatory action of insulin (302.1±2.1% stimulation by insulin alone *vs.* 346.1±10.6% insulin in the presence of the inhibitor; P=NS). Pertussis toxin did not have any effect on the basal TG synthesis at the concentration tested. This result indicates that the immediate post ASP receptor event does not involve $G\alpha_i$ subunit.

Intracellular signaling can occur because key proteins on the membrane cleave specific phospholipids into smaller diffusable molecules that convey the signal to other parts of the cell. Those proteins are known as phospholipases (PLC, PLD and PLA_2) and are activated in response to ligand binding the receptor. Although, to date, the inhibitors for $G\alpha_q$ and $G\alpha_{12/13}$ families are not yet identified, $G\alpha_q$ has been shown to activate phospholipase C (PLC), specifically, PLCβ isoform (153). Interestingly, PLCβ2 has been shown to also be activated by the Gβ/γ subunit (154) generated by the activated receptors of formyl Met-Leu-Phe/ C5aR family (C5L2, the ASP receptor, belongs to this gene family) (145). PLC, in turn, is a key enzyme involved in phosphatidyl inositol turnover and it catalyses the hydrolysis of phosphotidylinositol 4,5-biphosphate (PIP_2) generating two intracellular messengers, inositol 1,4,5-triphosphate (IP_3) and

diacylglycerol (DAG). IP_3 induces calcium mobilization from the endoplasmic reticulum and DAG activates protein kinase C (PKC) (155). Based on the experimental data from Baldo *et al*, biphasic increases in the intracellular DAG levels as well as PKC activation take place in cells exposed to ASP (109). This strongly suggests that PLC and/or possibly other phospholipases (PLD and PLA_2) may be involved in ASP signaling (156).

To evaluate the involvement of PLC we first tested the effect of U73122, a potent and widely used inhibitor of PLC action on phosphatidyl inositol hydrolysis. The results of these experiments are presented in Figure 2A. ASP (5 μM) stimulated TGS by 149.5±10.9% ($P<0.0003$ for ASP *vs.* basal TGS). In the presence of increasing concentrations of U73122 the ASP stimulatory effect was reduced by 90% at 10 μM U73122 ($P<0.004$ *vs.* ASP alone) and at 20 μM U73122 there was a complete loss of ASP stimulatory activity ($P<0.03$ *vs.* ASP alone). While treatment with U73122 resulted in a decrease of basal TG synthesis to 87.9±5.7% and 78.6±8.9% at 10 and 20 μM of the inhibitor, respectively (basal = 100%), these decreases were not significant. Insulin stimulated TG synthesis was 249.1±38.8% *vs.* basal and remained high at all concentrations of inhibitor tested (n=3). There was no significant effect of PLC inhibition on insulin-stimulated TGS. The results show that ASP treatment invokes PLC activation.

We then tested the involvement of phospholipase D (PLD) in the signaling of ASP. PLD hydrolyses phosphatidylcholine to phosphatidic acid (which can subsequently be converted into DAG) and choline which, in turn, are second

messengers themselves (157). As well, PLD has been demonstrated to be regulated by PKC (158). In fact, Baldo *et al* (109) has observed a second, prolonged raise in intracellular DAG, which indicates that phosphatidyl choline turnover may take place during ASP stimulation of TG synthesis. We evaluated activation of PLD in our system and the results are presented in Figure 2B. n-Butanol, a primary alcohol, which serves as a substrate for PLD in place of the phospholipid molecule generating phosphatidylalcohol instead of phosphatidic acid (159). Cells were first preincubated with increasing concentrations of n-butanol for 30 minutes at which time ASP was added and stimulation of TG synthesis was measured after 4 hours. At baseline ASP stimulated TGS was 165.1±11.1% ($P<0.0001$). The ASP effect was not inhibited at any n-butanol concentrations tested (Figure 2B). Interestingly, the basal levels of TG synthesis rose significantly in the presence of increasing concentrations of n-bulanol (100.0±3.0% without butanol *vs.* 304.1±33.9% with 0.6% n-butanol, $P< 0.0001$). At present it is not clear why basal TGS levels were increased with n-butanol, however, there is a possibility that n-butanol may stimulate another pathway (perhaps increasing glucose transport or inhibition of lipolysis, both of which could result in augmentation of TGS unrelated to ASP effects). Based on the presented experimental data PLD may not participate in ASP stimulation of TGS, however, this observation should be further explored with another inhibitor, which is specific for phosphatidic acid signaling.

Finally, the involvement of PLA_2 in ASP action was evaluated. Arachidonic and lysophosphatidic acid are 2 second messengers produced by the action of

PLA_2 (160). The inhibitor of phospholipase A_2 (PLA_2), isotetrandrine (specific for G-protein coupled PLA_2 (161)) was used and the results are shown in Figure 2C. Isotetrandrine was not effective in inhibiting the stimulatory effect of ASP on TGS. ASP stimulated TG synthesis was 190.3±14.7% ($P<0.0005$, *vs.* basal, n=3) and remained high upon addition of the inhibitor (209.5±20.9% at 10 µM, P=NS, n=3). Basal TGS levels were not affected by isotetrandrine treatment. However, since there exist a number of different PLA_2 groups, at this point we cannot rule out the involvement of members of the other PLA_2 families. These will have to be tested with additional, specific inhibitors.

The breakdown of inositol phospholipids (e.g. PIP_2) leading to the production of second messengers, IP_3 and DAG, is not the whole story in the regulation of intracellular signaling. A number of PIP exist in various phosphorylated forms that are convertible by phosphorylation and dephosphorylation steps. This has led to the hypothesis that some of these lipids are signaling molecules themselves. The kinase involved in the production of 3-phospho derivatives of the inositol lipids is phosphatidyl inositol 3 kinase (PI3K). PIP_2 and to a lesser extent PIP are precursors of phosphatidyl inositol 3,4,5-triphosphate and phosphatidyl inositol 3,4-biphosphate, respectively and are produced by the action of PI3K. Interestingly, recent evidence suggests that PI3K is not only activated by receptor tyrosine kinases but also by the G protein-coupled receptors (162). The lipids produced by the action of PI3K, in addition to being the main source of IP_3 and DAG, can also interact directly with specific intracellular enzymes to regulate their activity (163).

The involvement of PI3K in the signaling pathway of ASP was analyzed using two inhibitors. Wortmannin is a cell-permeable, irreversible inhibitor of the catalytic activity of PI3K at low nM concentrations (10nM - 100nM) but it may also inhibit other enzymes at higher nM concentrations. LY294002, on the other hand, is a reversible inhibitor of the catalytic site of PI3K but is specific for PI3K at concentrations of up to 50 μM. These inhibitors have been extensively used to demonstrate the role of PI3K in insulin action on glucose transport (164;165). Effect of both inhibitors was tested and the results are presented in Figure 3. Preincubation of 3T3-L1 preadipocytes for 30 min with either of the inhibitors alone was followed by addition of ASP or insulin and TGS was measured at 4 hours. As shown in Figure 3A, wortmannin inhibited the ASP stimulatory effect (258.3±24.7% ASP *vs.* basal, $P<0.0001$) by 70% (ASP + 100 nM wortmannin = 148.1±6.7%, $p<0.0001$). Inhibition was evident at all concentrations tested. The fact that inhibition was observed at low concentrations of wortmannin (25 nM) suggests that class I (class IA and IB) PI3K is involved (166) in ASP TG stimulatory action. This is interesting in view of the fact that class IB PI3K, p110γ, is activated by a stimulus received from G protein-coupled receptors. More specifically, Gβ/γ subunit has been shown to bind and activate p110γ (162;167). Wortmannin also inhibited 80% of insulin effect on TG synthesis (360.8±20.1% insulin without inhibitor *vs.* 156.1±6.4% insulin with 100 nM of wortmannin, $P<0.0001$). The involvement of PI3K in ASP action was further confirmed with experiments using LY294002. As shown in Figure 3B, ASP-stimulated TGS was 406.6±12.6% ($P<0.0001$). In this set of experiments ASP TG stimulatory effect

was much larger than usually seen, we believe that it is dependent on the particular batch of isolated ASP. In any case, this stimulation was reduced in the presence of increasing concentrations of this inhibitor and reached 179.2±42.5% (75% reduction in stimulation, $P<0.001$) and 116.9±24.0% (95% reduction in stimulation, $P<0.0001$) at 50 μM and 100 μM of LY294002, respectively.

The Plecstrin homology (PH) domain, a lipid-binding domain, is found in a vast array of intracellular proteins that act as primary effectors of the PI3K signaling system. By binding the PI3K lipid products (PIP_3, PIP_2), PH domain plays a critical role in coupling PI3K signals to downstream effectors and signaling pathways. A number of those are now characterized to a greater or lesser extent. Activation by PIP_3 has been shown for 3-phosphoinositide dependent protein kinase-1 (PDK-1) (168), Ras (169) and PKC (discussed below). PDK-1, in turn, can signal to a number of different targets, one of which is p70 S6 kinase (gene transcription/RNA translation pathway). On the other hand, PI3K activated Ras will activate mitogen-activated protein kinase / extracellular-signal-regulated kinase MAPK/ERK1/2 (cellular proliferation/differentiation pathways). We have targeted these two pathways by using two specific inhibitors, Rapamycin (specific inhibitor of mTOR and therefore p70 S6 kinase) and PD098059 (specific MAPK/ERK activation inhibitor) and the results are presented in Figure 4. Rapamycin (Figure 4A) did not inhibit ASP TGS stimulatory activity at any of the concentrations tested (166.4±9.2% *vs.* 161.2±15.1% ASP without *vs.* ASP with 2000 nM rapamycin; P=NS). On the other hand, there was significant inhibition observed with PD098059 at all of the

tested concentrations (244.3±16.1% ASP alone *vs.* 181.1±4.2% and 160.0±4.2% at 10 μM and at 50 μM of PD098059). This inhibition was 58% at 50 μM PD098059 ($P<0.0002$). These results indicate partial involvement of the MAP kinase pathway in the ASP action on TGS in 3T3-L1 preadipocytes. Certainly, it is interesting to see activation of MAPK/ERK signaling upon ASP exposure since involvement of this pathway has been implicated in induction of adipogenesis in 3T3-L1 preadipocytes (170). Moreover, several PKC family members have been implicated in the activation of MAPK pathway via phosphorylation of Raf.

Finally, there is now available evidence that a key event in PKC regulation is the phosphorylation of the newly synthesized PKC by PDK-1 (171). This is true for all the known classes of PKC (conventional, novel and atypical) (171-173). This would suggest that PKC activation is downstream of PI3K. In the present study PI3K is implicated in ASP signaling and we have previously demonstrated PKC involvement (109). To test if PKC is downstream of PI3K activation by ASP, we used a known PKC stimulator – phorbol 12-myristate 13-acetate (PMA). Baldo *et al* has demonstrated, that the effect of ASP on TG synthesis can be mimicked by PMA and, at least in human skin fibroblasts, the effect of PMA on TG synthesis was not additive to that of ASP (indicating involvement of the same PKC family) (109). Cells were incubated with or without wortmannin (100 nM) and 30 min later ASP, insulin or PMA were added for an additional 4 hours to measure TG synthesis. Figure 5 shows the results of this experiment. Although wortmannin inhibited ASP (74% reduction) and insulin (50% reduction) effect, the TG stimulatory action of PMA was the same with or without wortmannin

treatment (P=NS). This preliminary data seems to indicate that PKC acts downstream of PI3K as a mediator of ASP-stimulated lipid synthesis.

Although many possibilities exist (some of them shown in thin arrows) based on the results of the above experiments and those published by Baldo *et al* (109) we would like to propose the following scenario for the initial steps of ASP intracellular signaling (bold arrows, see Figure 6). Upon ASP binding to its receptor (C5L2, 7 transmembrane G protein-coupled receptor) activation of trimeric G protein ensues. In so doing Gα subunit dissociates from Gβ/γ subunit. Gβ/γ activates PI3K which phosphorylates PI at position 3 of inositol ring generating a phosphatidyl inositol 3,4,5-triphospate (PIP_3). This newly formed lipid activates PDK-1 which is necessary for phosphorylation of the PKC (PKC-P) and in so doing it causes the release of PKC-P into cytoplasm. Cytoplasmic PKC-P, still inactive, can be activated by intracellular Ca^{++} and/or DAG (except for the atypical isoforms, which seem to be activated by phosphorylation). PLC activation (which in our case could be activated through $G\alpha_q$ or Gβ/γ subunit) results in the release of IP_3 and DAG. IP_3 stimulates the release of Ca^{++} from the endoplasmic reticulum. Both Ca^{++} and DAG are now available for the activation of PKC. We have previously demonstrated that activation by ASP leads to transient increases in DAG, PKC activity and its translocation (109). Note that while PI3K and PLC are proposed to be activated in parallel, activation of both appears to be necessary since blockage of one or the other results in ASP inhibition.

Downstream of this we can only speculate as to how PKC activation leads to increased TG synthesis. We propose that ASP activates DGAT, which is specific to TG synthesis, since there is no increase in PL synthesis with ASP (personal communication). Two DGAT genes have recently been cloned (DGAT1 and DGAT2) (24;174) but there is little information yet available on their regulation. Based on circumstantial evidence, and similarities to other endoplasmic reticulum associated anabolic enzymes (ACAT, GPAT) (174;175) it is reasonable to assume that this enzyme might be activated through dephosphorylation. In this case, downstream of PKC, we would invoke activation of a phosphatase enzyme, leading to activation of DGAT.

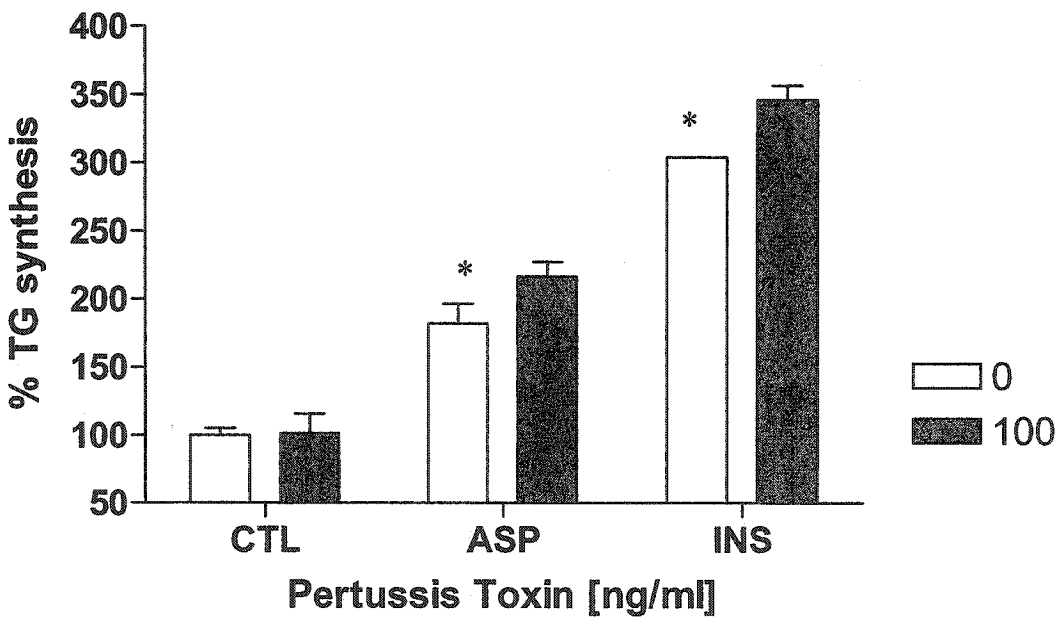

400
350
300
250
200
150
100
50
% TG synthesis
*
*
CTL
ASP
INS
Pertussis Toxin [ng/ml]
0
100

Figure 4.1 Effect of pertussis toxin on ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with 100 ng/ml of pertussis toxin for 4 hours and were then stimulated with 5 μM ASP or 100 nM insulin for an additional 4 hours. Pertussis toxin was present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis in each of the experiments performed (n=2 each performed in triplicate) and are presented as % TG stimulation, where basal (no addition and no treatment) was set as 100%. The data was analyzed using t-test and the significance was set at $p<0.05$. * $p<0.005$ with ASP (or insulin) as compared to basal.

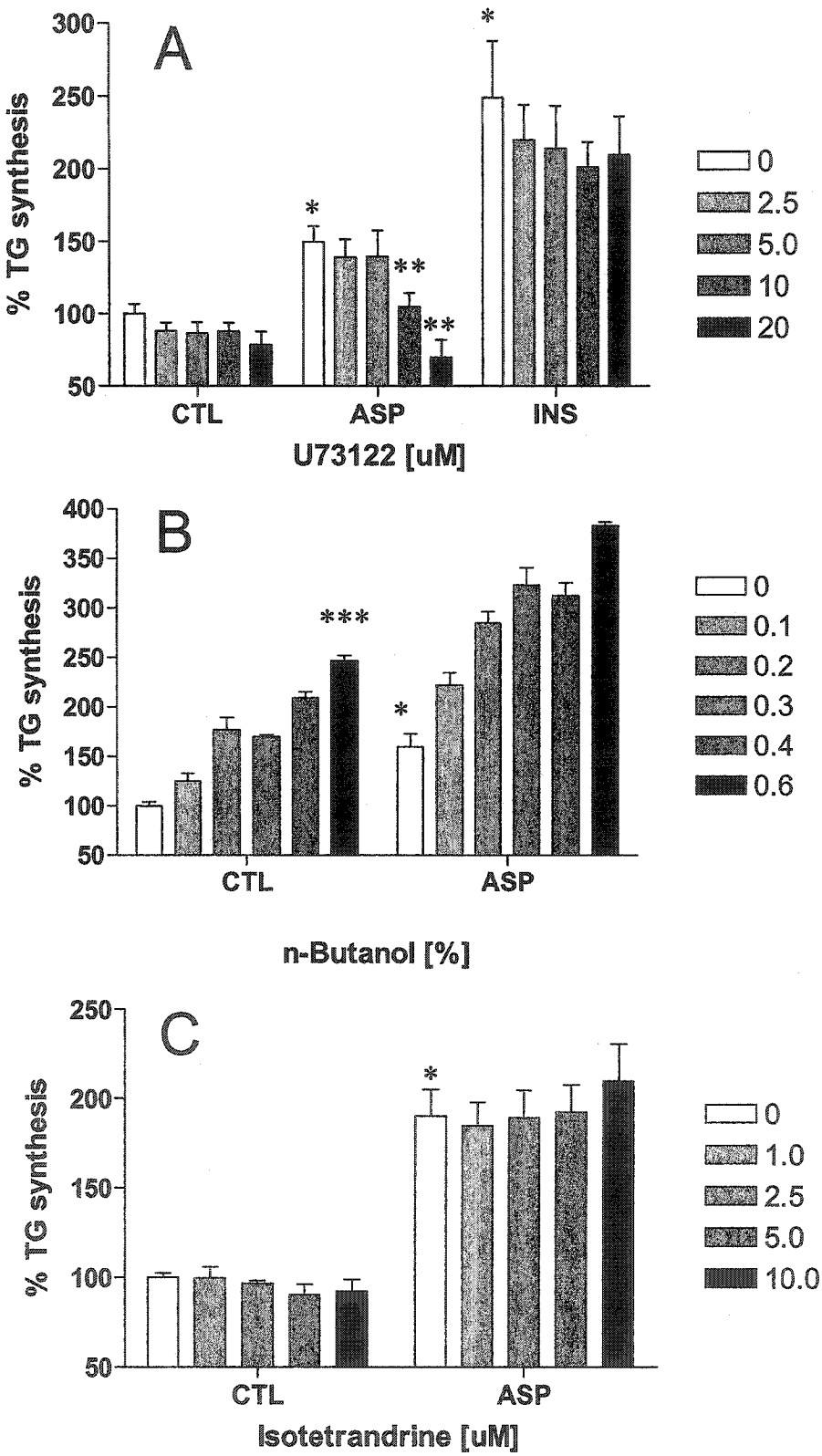
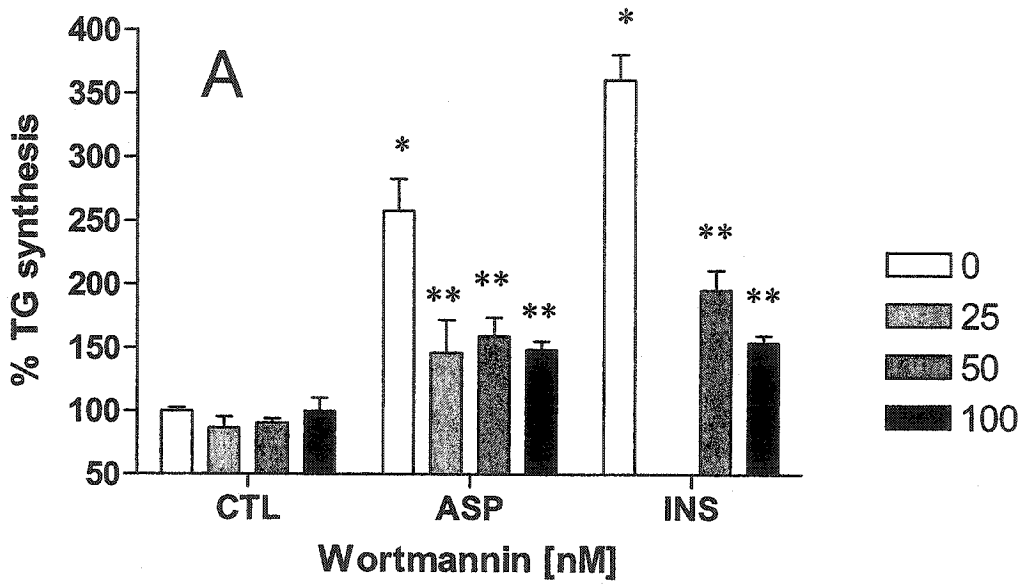

A
% TG synthesis
300
250
200
150
100
50
*
*
**
**
CTL
ASP
INS
U73122 [uM]
0
2.5
5.0
10
20
B
% TG synthesis
400
350
300
250
200
150
100
50
***
*
CTL
ASP
n-Butanol [%]
0
0.1
0.2
0.3
0.4
0.6
C
% TG synthesis
250
200
150
100
50
*
CTL
ASP
Isotetrandrine [uM]
0
1.0
2.5
5.0
10.0

Figure 4.2 Effect of phospholipase inhibition on ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with various concentrations of A) U73122 (PLC inhibitor), B) n-Butanol (a PLD substrate substitute) and C) isotetrandrine (G protein-coupled PLA_2 inhibitor) for 30 minutes and were then stimulated with 5 μM ASP or 100 nM insulin for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis in each of the experiments performed (n=3, each performed in duplicate or triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The data was analyzed using t-test and the significance was set at $p<0.05$. * $p<0.0005$ with ASP vs. basal; ** $p<0.003$ ASP with PLC inhibitor vs. ASP alone; *** $p<0.0001$ with 0.6% n-butanol vs. basal.


A
% TG synthesis
400
350
300
250
200
150
100
50
CTL
ASP
INS
Wortmannin [nM]
0
25
50
100

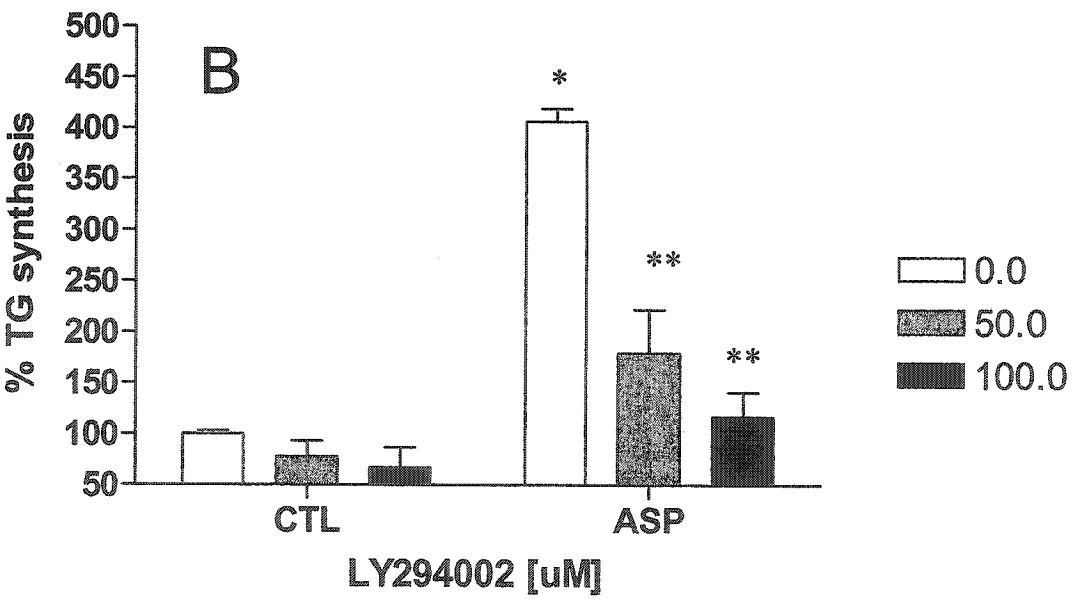
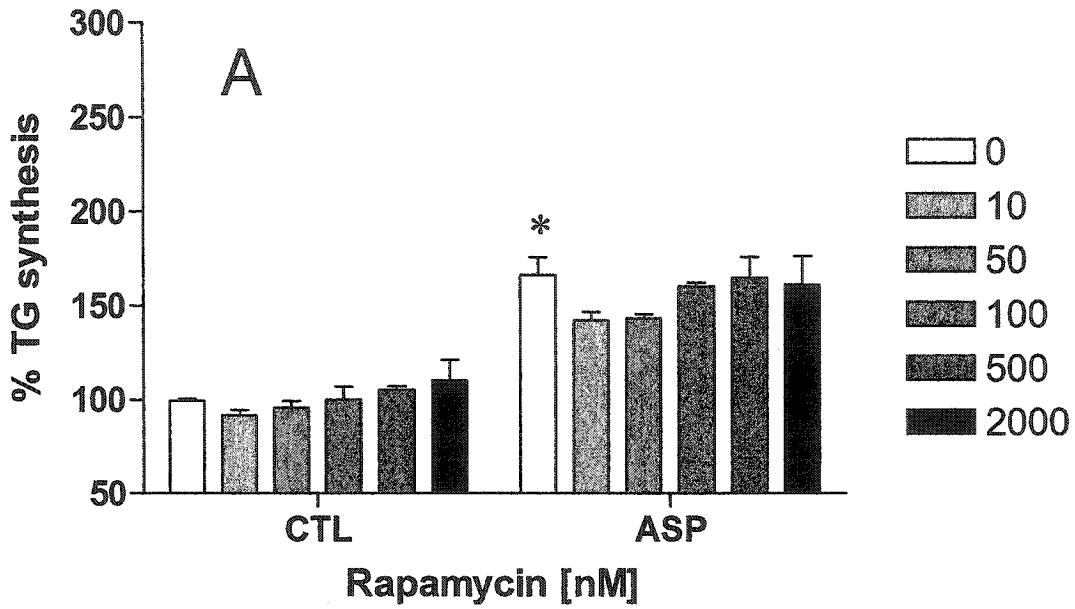

B
% TG synthesis
500
450
400
350
300
250
200
150
100
50
CTL
ASP
LY294002 [uM]
0.0
50.0
100.0

Figure 4.3 Effect of PI3K inhibition on ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with various concentrations of A) Wortmannin and B) LY294002 for 30 minutes and were then stimulated with 5 μM ASP or 100 nM insulin for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis in each of the experiments performed (n=7 for wortmannin and n=2 for LY294002, each performed in duplicate or triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The data was analyzed using t-test and the significance was set at $p<0.05$. * $p<0.0001$ for ASP (or insulin) treated cells vs. basal; ** $p<0.0001$ for ASP (or insulin) with wortmannin (or LY294002) vs. ASP alone.


A
300
250
200
150
100
50
% TG synthesis
*
CTL
ASP
Rapamycin [nM]
0
10
50
100
500
2000

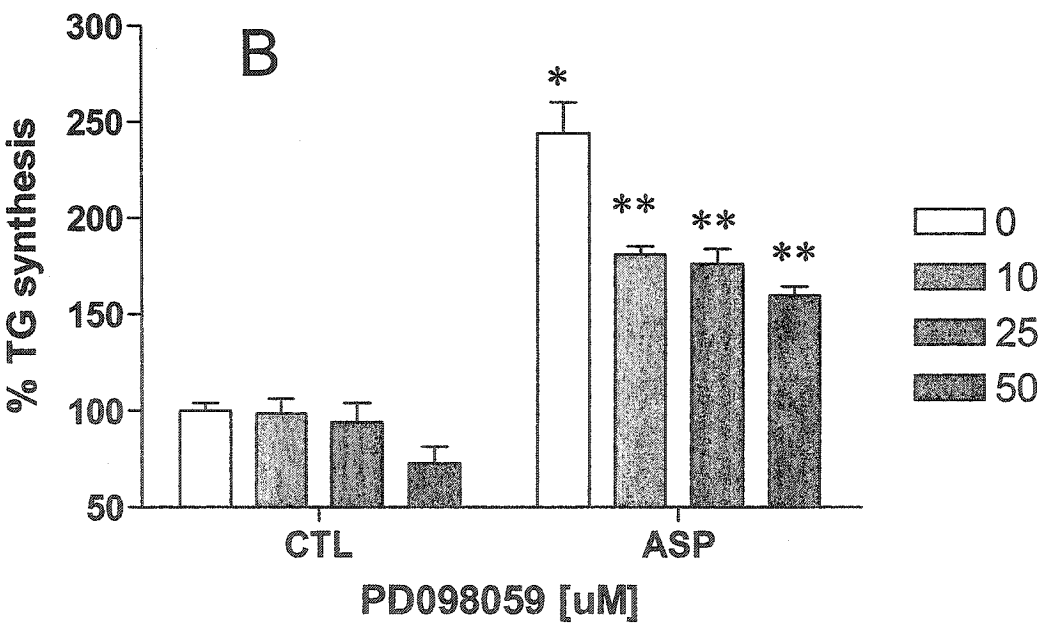

B
300
250
200
150
100
50
% TG synthesis
*
**
**
**
CTL
ASP
PD098059 [uM]
0
10
25
50

Figure 4.4 Effect of p70S6 kinase and MAPK/ERK1/2 pathways on ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with various concentrations of A) rapamycin (p70S6 kinase inhibitor) and B) PD098059 (MAPK/ERK inhibitor) for 30 minutes and were then stimulated with 5 μM ASP or 100 nM insulin for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis in each of the experiments performed (n=2 for each inhibitor, each performed in duplicate or triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The data was analyzed using t-test and the significance was set at $p<0.05$. * $p<0.005$ with ASP vs. basal; ** $p<0.0002$ ASP with PD098059 vs. ASP alone.

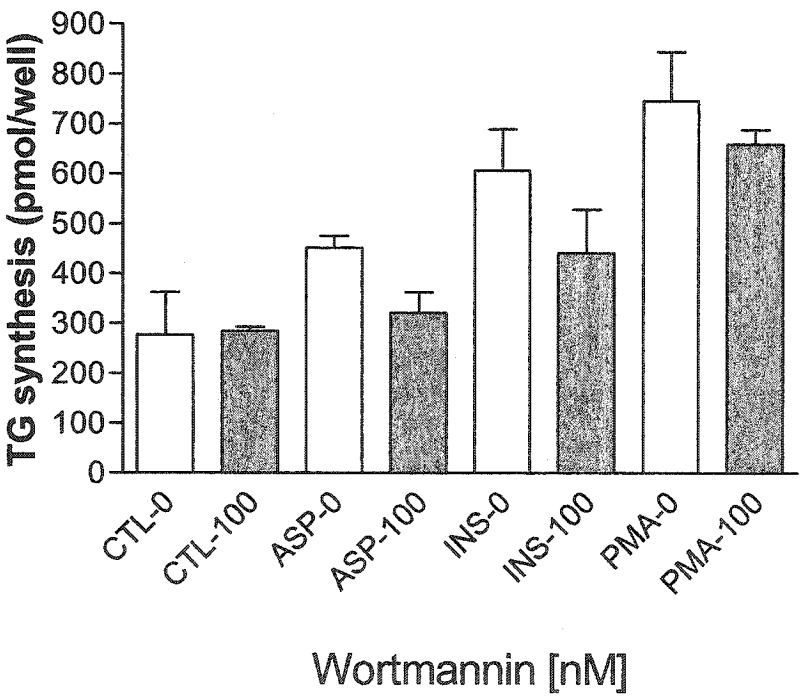

TG synthesis (pmol/well)
900
800
700
600
500
400
300
200
100
0
CTL-0
CTL-100
ASP-0
ASP-100
INS-0
INS-100
PMA-0
PMA-100
Wortmannin [nM]

Figure 4.5 Effect on TG synthesis inhibition with wortmannin in 3T3-L1 preadipocytes. Confluent cells were pretreated with 100 nM wortmannin for 30 minutes and the TG synthesis was then stimulated with 5 μM ASP, 100 nM insulin or 100 ng/ml PMA for an additional 4 hours. The inhibitor was present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results are presented as mean ± standard deviation (n=1). The data was analyzed using t-test and the significance was set at $P<0.05$.

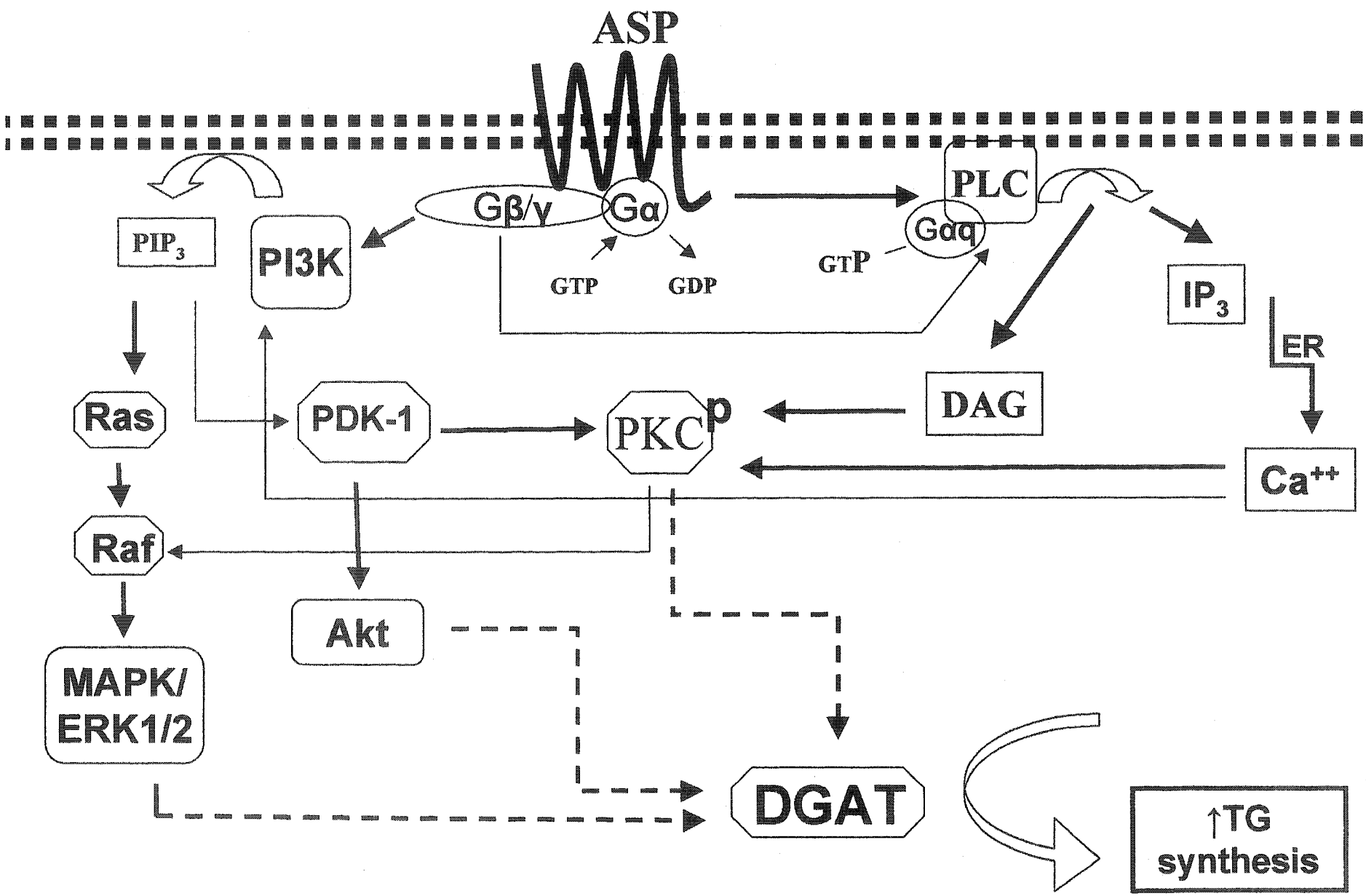

ASP
Gβ/γ
Gα
GTP
GDP
PLC
Gαq
GTP
PIP3
PI3K
IP3
ER
Ras
PDK-1
PKCp
DAG
Ca++
Raf
Akt
MAPK/
ERK1/2
DGAT
↑TG
synthesis

Figure 4.6 Proposed Intracellular Signaling Pathway for ASP Stimulated Triglyceride Synthesis.

Acylation stimulating protein (ASP), receptor for ASP (C5L2), phosphatidylinositol 4,5-biphosphate (PIP_2), phosphatidylinositol 3,4,5-triphosphate (PIP_3), inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), diacylglycerol acyltransferase (DGAT), triglyceride (TG), endoplasmic reticulum (ER), protein kinase C (PKC), phosphatidyl inositiol 3-kinase (PIP3K), phospholipase C (PLC), 3-phosphoinositide dependent protein kinase-1 (PDK-1), mitogen-activated protein kinase (MAPK/ERK1/2).

Chapter 5

General Summary

and

Concluding Remarks

5.1 SUMMARY

Understanding the physiological role and the mechanism of action of Acylation Stimulating Protein in the regulation of lipid metabolism is key to understanding the development of obesity and its related complications such as diabetes and cardiovascular disease. The work presented in this thesis has addressed a number of issues in this regard. Each chapter dealt with a different aspect of ASP: its action, its receptor and its signaling. We believe that the data obtained over the course of this Ph.D. study bring us closer to understanding how lipid metabolism is regulated at the cell level.

Although over the last several years, evidence has accumulated that ASP (C3adesArg) acutely influences lipid metabolism *in vitro* as well as *in vivo,* this is the first time that a novel, long-term effect of ASP on lipid metabolism has been demonstrated. Using 3T3-L1 and 3T3-F442A cell models we have shown that ASP treatment induces differentiation of preadipocytes into adipocytes. More specifically, the induced changes were observed at the metabolic and at the gene level and paralleled those previously published for insulin. Interestingly, the data generated by microarray analysis provided preliminary evidence that the very first molecular events are likely quite different between ASP and insulin. These observations are quite important in view of the fact that ASP production by human adipocytes is regulated by dietary fat (chylomicrons) and that it strongly correlates with plasma TG clearance. In humans, increased plasma levels of ASP (2-3 fold) have been shown in obese subjects, which decrease with fasting. Moreover, high affinity binding of ASP to adipose tissue plasma membranes

demonstrates that this is clearly one target of ASP. Although, in this study, we did not evaluate if C3a (an immediate ASP precursor) induces adipocyte differentiation we believe that the rapid conversion rates of C3a to ASP preclude C3a effects to be physiologically relevant. Thus, in a scenario where the body is being constantly bombarded with high caloric food intake, the consequence of increased and constant production of ASP will not only result in increased storage of fat in the form of TG but will also provide more adipocytes available to support this storage.

The second important issue addressed in this thesis was the identification of the ASP receptor. When these studies were initiated, evidence at that time indicated that ASP binding was specific and of high affinity (HSF and adipocytes). Although ASP (C3a desArg) is structurally related to C3a, C3a desArg does not bind to the C3a receptor. Data presented in Chapter 3 is the first report of the existence of a specific ASP receptor. We have identified C5L2 as the first receptor capable of ASP (C3a desArg) binding and shown that the binding characteristics of ASP on C5L2 are very similar to those observed in human skin fibroblasts (ASP responsive cells). Our studies also demonstrate that in RBL cells transfected with C5L2, C5L2 receptor binds more than one complement protein. C5a and C5adesArg bind with high affinity on one site and the other complement proteins bind a different, lower affinity site such that C3a=C4adesArg>C4a>ASP(C3adesArg). The meaning of this differential binding and how it relates to the acylation activity (TGS) is presently unclear. Additional experiments looking at the binding vs. activity with the complement proteins

using HEK cells (cells capable of high TG synthetic rates) transfected with C5L2 receptor will be done in the near future. We have demonstrated that at least in the cells responsive to the TG stimulatory actions of ASP 3T3 cells), none of the complement proteins stimulate TGS.

We have also demonstrated for the first time the expression of C5L2 in human skin fibroblasts, mouse 3T3-L1 preadipocytes and human adipose tissue (all cells responsive to ASP) but not on HEK cells (non-responders). On the other hand, we have shown that ASP (the bioactive molecule for TG synthesis) does not bind related C3a and C5a receptors. Although not addressed in this thesis, we already have preliminary experimental evidence that ASP stimulates TG synthesis in cells transfected with C5L2 and that C5L2 is internalized bound to β-arrestin indicating that the receptor is signals though G protein interaction (personal communication). Still, much remains to be learned about C5L2 participation in lipid metabolism. The identification of this receptor as the first ASP receptor paves the way to answering a number of experimental questions. For example, testing of differences in expression and protein levels of C5L2 in human adipose tissue from various depots will allow us to understand how, and if C5L2 participates in the development of obesity. Since fat accumulation in males and females is evidently different, it will be of great interest to see gender dependent differences in C5L2 expression. We have previously shown that binding of ASP to adipose tissue plasma membranes varies according to gender, obesity status and depot site. In addition, development of a C5L2 knockout

mouse will provide a new model to study the importance of this receptor in a physiological setting.

Finally, we have begun to identify the specific components of the ASP intracellular signaling pathway. These studies were undertaken to target various known enzymatic pathways. Chapter 4 shows, for the first time, the involvement of phospholipase C, PI3K and MAPK/ERK in the ASP stimulated TG synthesis. We have attempted to connect all the intracellular steps and present them in a coherent manner to be able to have a framework to draw from. We are well aware that other possibilities may exist as to how the specific intracellular pathways are connected and we will attempt to explore them further in the near future. The results are preliminary in that the data presented is based on the work with various inhibitors to pinpoint the various intracellular players and are the first stage in a multistage project. However, these findings are very interesting in view of the fact that to date little is known of TG synthesis regulation in adipocytes. It is with this generated knowledge that we can now use additional experimental techniques to prove and further these new findings. As well, these results bring us closer to our ultimate goal of understanding how activation of the ASP receptor, C5L2, couples to the regulation of diacylglycerol acyltransferase (DGAT), the last enzyme of the TG synthetic pathway, regulation of TG stores and possible participation in the development of obesity.

5.2 FUTURE WORK

As a result of the studies presented in this thesis there are a number of issues that will be addressed in the near future. Proving the functionality of C5L2 receptor in ASP TG stimulatory action is already being studied by using HEK-C5L2 stable transfectants. Conversly, by using interfering RNA technology we hope to inhibit endogenously expressed C5L2 and therefore the ASP effect. Furthermore, generation of C5L2 transgenic mice, C5L2 (-/-) will provide a system to study the contribution of C5L2 to the physiological regulation of lipid metabolism. We would expect that the resulting phenotype would be lean mice.

Linking ASP binding to C5L2 and its signaling pathway will be studied using a number of experimental techniques such as 1) binding of ASP to plasma membranes using GTP?S (in the presence of GTP?S binding of ASP will decrease), 2) ß-arrestin binding assay (these are receptor internalization studies that are presently being done to observe if ASP induces internalization of C5L2) and 3) using immunoprecipitation techniques along with specific antibodies to various members of G protein families (this will demonstrate which G protein couples with C5L2). Subsequently, we propose to use 1) immunoprecipitation techniques and 2) analysis of various intracellular lipids (PIP, LPA, PA, etc.) to confirm the results obtained with inhibitors. Deciphering the sequence of intracellular events and further characterizing the specific components of the second messenger pathway will also be addressed. More specifically, we would like to study which PKC isoform is activated by ASP and how DGAT is regulated (phosphorylation or dephosphorylation)?

Finally, if ASP induces adipocyte differentiation then it would be interesting to see which specific genes are up- or down regulated. Microarray analysis revealed a number of possible target genes that we can now study using various molecular biology techniques. Especially important, the findings from the studies on the ASP signaling pathway and ASP effects on adipocyte differentiation, which were done in the 3T3 preadipocyte cell model, should be extended to an *in vivo* system such as primary human preadipocytes and adipocytes, to demonstrate that the observed changes are relevant to normal human cell physiology.

5.3 CLAIMS OF ORIGINAL RESEARCH

The following experimental results and conclusions represent original contribution to science:

1. ASP, an adipose tissue produced hormone, induces differentiation in the *in vitro* cell models (3T3-L1 and 3T3-F442A) indicating that adipose tissue can self-regulate its own mass at the metabolic as well as molecular level.
2. The very early events in ASP induced adipocyte differentiation vary from those observed with insulin implying participation of different intracellular pathways.
3. The first receptor for ASP, C5L2, has been identified and shown to be expressed in human skin fibroblasts, human adipose tissue and 3T3-L1 preadipocytes.
4. ASP and C3a binding characteristics in human skin fibroblasts have been shown to be similar, however, ASP, which is bioactive for TGS, does not bind to the C3a receptor.
5. ASP does not stimulate TGS in U937, U937cAMP, HEK and HEK-C3aR cells nor do C4a, C4adesArg, C5a, C5adesArg stimulate TGS in 3T3-L1 preadipocytes (ASP responsive cells).
6. ASP stimulation of TG synthetic pathway involves participation of PLC, PI3K and MAPK but not PLA_2, PLD or p70 S6 kinase.
7. ASP activation of PKC is downstream of PI3K activation.

5.4 GENERAL REFERENCES

1. **Tso P, Balint JA** 1986 Formation and transport of chylomicrons by enterocytes to the lymphatics. American Journal of Physiology 250:G715-G726

2. **Fojo SS, Law SW, Spreecher DL, Gregg RE, Baggio G, Brewer HB, Jr.** 1984 Analysis of the apoC-II gene in apoC-II deficient patients. Biochem Biophys Res Commun 124:308-313

3. **Olivecrona T, Hultin M, Bergo M, Olivecrona G** 1997 Lipoprotein lipase: regulation and role in lipoprotein metabolism. Proceedings of the Nutrition Society 56:723-729

4. **Chajek-Shaul T, Friedman G, Bengtsson-Olivecrona G, Vlodavsky I, Bar-Shavit R** 1990 Interaction of lipoprotein lipase with subendothelial extracellular matrix. Biochim Biophys Acta 1042:168-175

5. **Dani C, Amri E, Bertrand BG, *et al*** 1990 Expression and regulation of pob24 and lipoprotein lipase genes during adipose conversion. Journal of Cellular Biochemistry 43:103-110

6. **Saxena U, Goldberg IJ** 1990 Interaction of lipoprotein lipase with glycosaminoglycans and apolipoproteins C-II: Effects of free fatty acids. Biochimica et Biophysica Acta 1043:161-168

7. **Farese RV, Jr., Yost TJ, Eckel RH** 1991 Tissue-specific regulation of lipoprotein lipase activity by insulin/glucose in normal-weight humans. Metabolism 40:214-216

8. **Smith LC, Pownall HJ, Gotto AM, Jr.** 1978 The plasma lipoproteins: structure and metabolism. Annu Rev Biochem 47:751-757

9. **Brasaemle DL, Rubin B, Harten IA, Gruia-Gray J, Kimmel AR, Londos C** 2000 Perilipin A increases triacylglycerol storage by decreasing the rate of triacylglycerol hydrolysis. Journal of Biological Chemistry 275:38486-38493

10. **Stahl A, Evans JG, Pattel S, Hirsch D, Lodish HF** 2002 Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. Dev Cell 2:477-488

11. **Hamilton JA** 1998 Fatty acid transport: difficult or easy? Journal of Lipid Research 39:467-481

12. **Stahl A, Gimeno RE, Tartaglia LA, Lodish HF** 2001 Fatty acid transport proteins: a current view of a growing family. Trends Endocrinol Metab 12:266-273

13. **Brecher P** 1983 The interaction of long-chain acyl CoA with membranes. Molecular & Cellular Biochemistry 57:3-15

14. **Igal RA, Wang P, Coleman RA** 1997 Triacsin C blocks de novo synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into

phospholipid: evidence for functionally separate pools of acyl-CoA. Biochem J 324:529-534

15. **Oelkers P, Behari A, Cromley D, Billheimer JT, Sturley SL** 1998 Characterization of two human genes encoding acyl coenzyme A:cholesterol acyltransferase-related enzymes. Journal of Biological Chemistry 273:26765-26771

16. **Ganesh Bhat B, Wang P, Kim JH, Black TM, Lewin TM, Fiedorek FTJr, Coleman RA** 1999 Rat sn-glycerol-3-phosphate acyltransferase: molecular cloning and characterization of the cDNA and expressed protein. Biochim Biophys Acta 1439:415-423

17. **Smith SJ, Cases S, Jensen DR, Chen HC, Sande E, Tow B, Sanan DA, Raber J, Eckel RH, Farese RVJr** 2000 Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. Nature Genetics 25:87-90

18. **Igal RA, Wang S, Gonzalez-Baro M, Coleman RA** 2001 Mitochondrial glycerol phosphate acyltransferase directs the incorporation of exogenous fatty acids into triacylglycerol. J Biol Chem 276:42205-42212

19. **Coleman RA, Bell RM** 1980 Selective changes in enzymes of the sn-glycerol 3-phosphate and dihydroxyacetone-phosphate pathways of triacylglycerol biosynthesis during differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 255:7681-7687

20. **Eberhardt C, Gray PW, Tjoelker LW** 1997 Human lysophosphatidic acid acyltransferase. cDNA cloning, expression, and localization to chromosome 9q34.3. Journal of Biological Chemistry 272:20299-20305

21. **West J, Tompkins CK, Balantac N, Nudelman E, Meengs B, White T, Bursten S, Coleman J, Kumar A, Singer JW, Leung DW** 1997 Cloning and expression of two human lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. DNA & Cell Biology 16:691-701

22. **Coleman RA, Reed BC, Mackall JC, Lane MD, Lane MD, Bell RM** 1978 Selective changes in microsomal enzymes of triacylglycerol phosphatidylcholine, and phosphatidylethanolamine biosynthesis during differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 253:7256-7261

23. **Tijburg LBM, Geelen MJH, van Golde LMG** 2003 Regulation of the biosynthesis of triglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. Biochim Biophys Acta 1004:1-19

24. **Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR, Sande E, Novak S, Collins C, Welch CB, Lusis AJ, Eriksson SJ, Farese RVJr** 1998 Identification of a gene encoding an acylCoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proceedings of the National Academy of Sciences of the United States of America 95:13018-13023

25. **Sarabia V, Ramlal T, Klip A** 1990 Glucose uptake in human and animal muscle cells in culture. Biochemistry & Cell Biology 68:536-542

26. **Shepherd PR, Gnudi L, Tozzo E, Yang H, Leach F, Kahn BB** 1993 Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. Journal of Biological Chemistry 268:22243-22246

27. **Galton DJ** 1968 Lipogenesis in human adipose tissue. Journal of Lipid Research 9:19-26

28. **Fredrikson G, Tornqvist H, Belfrage P** 2003 Hormone-sensitive lipase and monoacylglycerol lipase are both required for complete degradation of adipocyte triacylglycerol. Biochim Biophys Acta 876:288-293

29. **Griffin J, Ojeda S** 1996 Textbook of Endocrine Physiology. Oxford University Press, New York, 3rd edn

30. **Belfrage P, Fredrikson G, Olsson H, Stralfors P** 1984 Regulation of adipose tissue lipolysis through reversible phosphorylation of hormone-sensitive lipase. Adv Cyclic Nucleotide Protein Phosphorylation Res 17:351-359

31. **Stralfors P, Belfrage P** 2003 Phosphorylation of hormone-sensitive lipase by cyclic AMP-dependent protein kinase. Journal of Biological Chemistry 258:15146-15152

32. **Spiegelman B, Flier JS** 2001 Obesity and the regulation of energy balance. Cell 104:531-543

33. **Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ, Gorenstein D, Chen KH, Chan L** 2000 Absence of perilipin results in leanness and reverses obesity in Lepr(*db/db*) mice. Nat Genet 26:474-479

34. **Kono T, Robinson FW, Sarver JA** 1975 Insulin-sensitive phosphodiesterase. Its localization, hormonal stimulation, and oxidative stabilization. Journal of Biological Chemistry 250:7826-7835

35. **Gregoire FM, Smas CM, Sul HS** 1998 Understanding adipocyte differentiation. Physiological Reviews 78:783-809

36. **MacDougald OA, Lane MD** 1995 Transcriptional regulation of gene expression during adipocyte differentiation. Annu Rev Biochem 64:345-373

37. **Entenmann G, Hauner H** 1996 Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am J Physiol 270:C1011-C1016.

38. **Selvarajan S, Lund LR, Takeuchi T, Craik CS, Werb Z** 2001 A plasma kallikrein-dependent plasminogen cascade required for adipocyte differentiation. nature cell biology 3:267-275

39. **Morrison RF, Farmer SR.** 2000 Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr 130:3116S-3121S

40. **Rosen ED, Spiegelman BM** 2000 Molecular regulation of adipogenesis. Annu Rev Cell Dev Giol 16:145-171

41. **Kim JB, Spiegelman BM** 1996 ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev 10:1096-1107

42. **Tong Q, Dalgin GXH, Ting CN, Leiden JM** 2000 Function of GATA transcription factors in preadipocyte-adipocyte transition. Science 290:134-138

43. **Reusch JE, Colton LA, Klemm DJ** 2000 CREB activation induces adipogenesis in 3T3-L1 cells. Mol Cell Biol 20:1008-1020

44. **Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA** 2001 The Adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. Am J Physiol Endo Metab 280:E827-E847

45. **Gregoire FM, Smas CM, Sul HS.** 1998 Understanding adipocyte differentiation. Physiol Rev 78:783-809

46. **van Dijk G** 2001 The role of leptin in the regulation of energy balance and adiposity. J Neuroendocrinol 13:913-921

47. **Brabant G, Nave H, Mayr B, Behrend M, Van Harmelen V, Arner P** 2002 Secretion of free and protein-bound leptin from subcutaneous adipose tissue of lean and obese women. J Clin Endocrinol Metab 87:3966-3970

48. **Bryson JM, Phuyal JL, Swan V, Caterson ID** 1999 Leptin has acute effects on glucose and lipid metabolism in both lean and gold thioglucose-obese mice. Am J Physiol 277:E417-E422

49. **Fruhbeck G, Aguado M, Gomez-Ambrosi J, Martinez JA** 1998 Lipolytic effect of *in vivo* leptin administration on adipocytes of lean and *ob/ob* mice, but not *db/db* mice. Biochemical and Biophysical Research Communications 250:99-102

50. **Sarmiento U, Benson B, Kaufman S, Ross L, Qi MSS, DiPalma C** 1997 Morphologic and molecular changes induced by recombinant human leptin in the white and brown adipose tissues of C57BL/6 mice. Lab Invest 77:243-256

51. **Ramsay TG** 2001 Porcine leptin alters insulin inhibition of lipolysis in porcine adipocytes *in vitro*. J Anim Sci 79:653-657

52. **Fruhbeck G, Gomez-Ambrosi J, Salvador J** 2001 Leptin-induced lipolysis opposes the tonic inhibition of endogenous adenosine in white adipocytes. FASEB J 15:333-340

53. **Aprath-Husmann I, Rohrig K, Gottschling-Zeller H, Skurk T, Scriba D, Birgel M, Hauner H** 2001 Effects of leptin on the differentiation and

metabolism of human adipocytes. Int J Obes Relat Metab Disord 25:1465-1470

54. **Hube F, Hauner H** 1999 The role of TNF-alpha in human adipose tissue: prevention of weight gain at the expense of insulin resistance. Horm Metab Res 31:626-631

55. **Szalkowski D, White-Carrington S, Berger J, Zhang B** 1995 Antidiabetic thiazolidinediones block the inhibitory effect of tumor necrosis factor-alpha on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells. Endocrinology 136:1474-1481

56. **Kawakami M, Watanabe N, Ogawa H, Kato A, Sando H, Yamada N, Murase T, Takaku F, Shibata S, Oda T** 1989 Cachectin/TNF kills or inhibits the differentiation of 3T3-L1 cells according to developmental stage. J Cell Physiol 138:1-7

57. **Mattacks CA, Pond CM** 1999 Interactions of noradrenalin and tumour necrosis factor alpha, interleukin 4 and interleukin 6 in the control of lipolysis from adipocytes around lymph nodes. Cytokine 11:334-346

58. **Doerrler W, Feingold KR, Grunfeld C** 1994 Cytokines induce catabolic effects in cultured adipocytes by multiple mechanisms. Cytokine 6:478-484

59. **Lyle RE, Richon VM, McGehee REJr** 1998 TNFalpha desrupts mitotic clonal expression. Biochem Biophys Res Commun 247:373-378

60. **Petruschke T, Hauner H** 1993 Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J Clin Endocrin Met 76:742-747

61. **Sethi JK, Xu H, Uysal KT, Wiesbrock SM, Scheja L, Hotamisligil GS** 2000 Characterization of receptor-specific TNFalpha functions in adipocyte cell lines lacking type 1 and 2 TNF receptors. FEBS Letters 469:77-82

62. **Coppack SW** 2001 Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc 60:349-356

63. **Engelman JA, Berg AH, Lewis RY, Lisanti MP, Scherer PE** 2000 Tumor necrosis factor alpha-mediated insulin resistance, but not dedifferentiation, is abrogated by MEK1/2 inhibitors in 3T3-L1 adipocytes. Biol Trace Elem Res 74:11-21

64. **Mohamed-Ali V, Pinkney JH, Coppack SW** 1998 Adipose tissue as an endocrine and paracrine organ. International Journal of Obesity and Related Metabolic Disorders 22:1145-1158

65. **Chrousos GP, Vgontzas AN, Papanicolaou DA, Bixler EO, Kales A, Tyson K, Chrousos GP** 1997 Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. Journal of Clinical Endocrinology and Metabolism 82:1313-1316

66. **Petruschke T, Rohrig K, Hauner H** 1994 Transforming growth factor beta (TGF-beta) inhibits the differentiation of human adipocyte precursor cells in

primary culture. International Journal of Obesity and Related Metabolic Disorders 18:532-536

67. **Sul HS, Smas C, Mei B, Zhou L** 2000 Function of pref-1 as an inhibitor of adipocyte differentiation. Int J Obes Relat Metab Disord 24:S15-S19

68. **Karlesson C, Lindell K, Ottosson M, Sjostrom L, Carlsson B, Carlsson LM** 1998 Human adipose tissue expresses angiotensinogen and enzymes required for its conversion to angiotensin II. J Clin Endocrinol Metab 83:3925-3929

69. **Jones BH, Standridge MK, Moustaid N** 1997 Angiotensin II increases lipogenesis in 3T3-L1 and human adipose cells. Endocrinology 138:1512-1519

70. **Saint-Marc P, Kozak LP, Ailhaud G, Darimont C, Negrel R** 2001 Angiotensin II as a trophic factor of white adipose tissue: stimulation of adipose cell formation. Endocrinology 142:487-492

71. **Darimont C, Vassaux G, Ailhaud G, Negrel R** 1994 Differentiation of preadipose cells: paracrine role of prostacyclin upon stimulation of adipose cells by angiotensin-II. Endocrinology 135:2030-2036

72. **Massiera F, Seydoux J, Geloen A, Quignard-Boulange A, Turban S, Saint-Marc P, Fukamizu A, Negrel R, Ailhaud G, Teboul M** 2001 Angiotensinogen-deficient mice exhibit impairment of diet-induced weight

gain with alteration in adipose tissue development and increased locomotor activity. Endocrinology 142:5220-5225

73. **Mallow H, Trindl A, Loffler G** 2000 Production of angiotensin II receptors type one (AT1) and type two (AT2) during the differentiation of 3T3-L1 preadipocytes. Horm Metab Res 32:500-503

74. **Yang YK, Ollmann MM, Wilson BD, Dickinson C, Yamada T, Barsh GS, Gantz I** 1997 Effects of recombinant agouti-signaling protein on melanocortin action. Mol Endocrinol 11:274-280

75. **Boston BA, Cone RD** 1996 Characterization of melanocortin receptor subtype expression in murine adipose tissues and in the 3T3-L1 cell line. Endocrinology 137:2043-2050

76. **Yen TT, Gill AM, Frigeri LG, Barsh GS, Wolff GL** 1994 Obesity, diabetes, and neoplasia in yellow A(vy)/- mice: ectopic expression of the agouti gene. FASEB J 8:479-488

77. **Mynatt R, Stephens JM** 2001 Agouti regulates adipocyte transcription factors. Am J Physiol Cell Physiol 280:C961

78. **Jones BH, Kim JH, Zemel MB, Woychik RP, Michaud EJ, Wilkison WO, Moustaid N** 1996 Upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. American Journal of Physiology 270:E192-E196

79. **Kwon HY, Bultman SJ, Loffler C, Chen WJ, Furdon PJ, Powell JG, Usala AL, Wilkison W, Hansmann I, Woychik RP** 1994 Molecular structure and chromosomal mapping of the human homolog of the agouti gene. Proceedings of the National Academy of Sciences of the United States of America 91:9760-9764

80. **Xue B, Zemel MB** 2000 Relationship between human adipose tissue agouti and fatty acid synthase (FAS). J Nutr 130:2478-2481

81. **Cianflone K, Rodriguez MA, Walsh M, Vu H, Sniderman AD** 1988 The effect of a plasma protein fraction on lipid synthesis in cultured skin fibroblasts from normals and patients with hyperapobetalipoproteinemia. Clinical & Investigative Medicine 11:99-107

82. **Baldo A, Sniderman AD, St-Luce S, Avramoglu RK, Maslowska M, Hoang B, Monge JC, Bell A, Mulay S, Cianflone K** 1993 The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. Journal of Clinical Investigation 92:1543-1547

83. **Hugli TE** 1989 Structure and function of C3a anaphylatoxin. Current Topics in Microbiology and Immunology 153:181-208

84. **Schreiber RD, Muller-Eberhard HJ** 1978 Assembly of the cytolytic alternative pathway of complement from 11 isolated plasma proteins. Journal of Experimental Medicine 148:1722-1727

85. **Lesavre PH, Hugli TE, Esser AF, Muller-Eberhard HJ** 1979 The alternative pathway C3/C5 convertase: chemical basis of factor B activation. Journal of Immunology 123:529-534

86. **Campbell WD, Lazoura E, Okada N, Okada H** 2002 Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. Microbiology and Immunology 46:131-134

87. **Choy LN, Rosen BS, Spiegelman BM** 1992 Adipsin and an endogenous pathway of complement from adipose cells. Journal of Biological Chemistry 267:12736-12741

88. **Cianflone K, Roncari DAK, Maslowska M, Baldo A, Forden J, Sniderman AD** 1994 The adipsin/acylation stimulating protein system in human adipocytes: Regulation of triacylglycerol synthesis. Biochemistry 33:9489-9495

89. **Cianflone K, Maslowska M** 1995 Differentiation induced production of ASP in human adipocytes. European Journal of Clinical Investigation 25:817-825

90. **Murray I, Parker RA, Kirchgessner TG, Tran J, Zhang ZJ, Westerlund J, Cianflone K** 1997 Functional bioactive recombinant Acylation Stimulating Protein is distinct from C3a anaphylatoxin. J Lipid Res 38:2492-2501

91. **Maslowska M, Vu H, Phelis S, Sniderman AD, Rhode BM, Blank D, Cianflone K** 1999 Plasma acylation stimulating protein, adipsin and lipids in non-obese and obese populations. Euro J Clin Inv 29:679-686

92. **Cianflone K, Sniderman AD, Kalant D, Marliss EB, Gougeon R** 1995 Response of plasma ASP to a prolonged fast. International Journal of Obesity 19:604-609

93. **Sniderman AD, Cianflone K, Eckel RH** 1991 Levels of acylation stimulating protein in obese women before and after moderate weight loss. International Journal of Obesity 15:333-336

94. **Faraj M, Jones P, Sniderman AD, Cianflone K** 2001 Enhanced dietary fat clearance in post-obese women. Journal of Lipid Research 42:571-580

95. **Saleh J, Summers LKM, Cianflone K, Fielding BA, Sniderman AD, Frayn KN** 1998 Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue *in vivo* in the postprandial period. J Lipid Res 39:884-891

96. **Maslowska M, Scantlebury T, Germinario R, Cianflone K** 1997 Acute *in vitro* production of ASP in differentiated adipocytes. J Lipid Res 38:21-31

97. **Scantlebury T, Maslowska M, Cianflone K** 1998 Chylomicron specific enhancement of Acylation Stimulating Protein (ASP) and precursor protein C3 production in differentiated human adipocytes. J Biol Chem 273:20903-20909

98. **Scantlebury T, Sniderman AD, Cianflone K** 2001 Retinoic Acid Regulation of Acylation Stimulating Protein (ASP) and complement C3 in Human Adipocytes. Biochemistry Journal 356:445-452

99. **Sadur CN, Eckel RH** 1982 Insulin stimulation of adipose tissue lipoprotein lipase. Use of the euglycemic clamp technique. Journal of Clinical Investigation 69:1119-1125

100. **Sooranna S, Saggerson E** 1975 Studies on the role of insulin in the regulation of glyceride synthesis in rat epididymal adipose tissue. Biochemistry 150:441-451

101. **Stralfors P, Honnor RC** 1989 Insulin-induced dephosphorylation of hormone-sensitive lipase. Correlation with lipolysis and cAMP-dependent protein kinase activity. Eur J Biochem 182:379-385

102. **Cianflone K, Maslowska M, Sniderman AD** 1999 Acylation stimulating protein (ASP), an adipocyte autocrine: new directions. Semin Cell Dev Biol 10:31-41

103. **Cianflone K** 1997 The Acylation Stimulating Protein Pathway: Clinical Implications. Clinical Biochemistry 30:301-312

104. **Yasruel Z, Cianflone K, Sniderman AD, Rosenbloom M, Walsh M, Rodriguez MA** 1991 Effect of acylation stimulating protein on the triacylglycerol synthetic pathway of human adipose tissue. Lipids 26:495-499

105. **Germinario R, Sniderman AD, Manuel S, Pratt S, Baldo A, Cianflone K** 1993 Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation stimulating protein. Metabolism 42:574-580

106. **Maslowska M, Sniderman AD, Germinario R, Cianflone K** 1997 ASP stimulates glucose transport in cultured human adipocytes. Int J Obes Relat Metab Disord 21:261-266

107. **Tao Y, Cianflone K, Sniderman AD, Colby-Germinario SP, Germinario RJ** 1997 Acylation-stimulating protein (ASP) regulates glucose transport in the rat L6 muscle cell line. Biochimica et Biophysica Acta 1344:221-229

108. **Van Harmelen V, Reynisdottir S, Cianflone K, Degerman E, Hoffstedt J, Nilsell K, Sniderman A, Arner P** 1999 Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. Journal of Biological Chemistry 274:18243-18251

109. **Baldo A, Sniderman AD, St.Luce S, Zhang XJ, Cianflone K** 1995 Signal transduction pathway of acylation stimulating protein: Involvement of protein kinase C. Journal of Lipid Research 36:1415-1426

110. **Murray I, Sniderman AD, Cianflone K** 1999 Enhanced triglyceride clearance with intraperitoneal human acylation stimulating protein (ASP) in C57Bl/6 mice. American Journal of Physiology: Endocrinology and Metabolism 277:E474-E480

111. **Murray I, Sniderman AD, Cianflone K** 1999 Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. J Lipid Res 40:1671-1676

112. **Saleh J, Sniderman AD, Cianflone K,** Postprandial triacylglycerol clearance in *ob/ob* and *db/db* mice: physiological effects of ASP in mouse models of obesity and hypertriglyceridemia. p S183 (Abstract)

113. **Murray I, Havel PJ, Sniderman AD, Cianflone K** 2000 Reduced body weight, adipose tissue, and leptin levels despite increased energy intake in female mice lacking acylation-stimulating protein. Endocrinology 141:1041-1049

114. **Xia Z, Sniderman AD, Cianflone K** 2002 Acylation-stimulating Protein (ASP) Deficiency Induces Obesity Resistance and Increased Energy Expenditure in *ob/ob* Mice. J Biol Chem 277:45874-45879

115. **Murray I, Kohl J, Cianflone K** 1999 Acylation stimulating protein (ASP): structure:function determinants of cell surface binding and triacylglycerol synthetic activity. Biochem J 342:41-48

116. **Saleh J, Christou N, Cianflone K** 1999 Regional specificity of ASP binding in human adipose tissue. American Journal of Physiology 276:E815-E821

117. **Zhang XJ, Cianflone K, Genest J, Sniderman AD** 1998 Plasma acylation stimulating protein (ASP) as a predictor of impaired cellular biological response to ASP in patients with hyperapoB. European Journal of Clinical Investigation 28:730-739

118. **Sato R, Imanaka T, Takano T** 1990 The effect of HMG-CoA reductase inhibitor (CS-514) on the synthesis and secretion of apolipoproteins B and A-1 in the human hepatoblastoma Hep G2. Biochimica et Biophysica Acta 1042:36-41

119. **Klos A, Bank S, Gietz C, Bautsch W, Kohl J, Burg M, Kretzschmar T** 1992 C3a receptor on Dibutyryl-cAMP-differentiated U937 cells and human neutrophils: The human C3a receptor characterized by functional responses and I-C3a binding. Biochemistry 31:11274-11282

120. **Fischer WH, Hugli TE** 1997 Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. Journal of Immunology 159:4279-4286

121. **Takabayashi T, Vannier E, Clark BD, Margolis NH, Dinarello CA, Burke JF, Gelfand JA** 1996 A new biologic role for C3A and C3A desarg - regulation of TNF-ALPHA and IL-1-Beta synthesis. Journal of Immunology 156:3455-3460

122. **Showell HJ, Glovsky MM, Ward PA** 1982 C3a-induced lysosomal enzyme secretion from human neutrophils: lack of inhibition by f met-leu-phe antagonists and inhibition by arachidonic acid antagonists. International Archives of Allergy & Applied Immunology 67:227-232

123. **Takafuji S, Tadokoro K, Ito K, Dahinden CA** 1994 Degranulation from human eosinophils stimulated with C3a and C5a. International Archives of Allergy & Immunology 104:27-29

124. **Volanakis JE** 1990 Participation of C3 and its ligands in complement activation. Current Topics in Microbiology and Immunology 153:1-21

125. **Stimler NP, Bloor CM, Hugli TE** 1983 C3a-induced contraction of guinea pig lung parenchyma: role of cyclooxygenase metabolites. Immunopharmacology 5:251-257

126. **Fukuoka Y, Tachibana T, Yasui A** 1994 Anaphylatoxin C3a induces rapid protein phosphorylation in guinea pig platelets. Immunopharmacology 28:95-104

127. **Zwirner J, Gotze O, Sieber A, Kapp A, Begemann G, Zuberbier T, Werfel T** 1998 The human mast cell line HMC-1 binds and responds to C3a but not C3a(desArg). Scandinavian Journal of Immunology 47:19-24

128. **Zwirner J, Werfel T, Wilken HC, Theile E, Gotze O** 1998 Anaphylatoxin C3a but not C3a(desArg) is a chemotaxin for the mouse macrophage cell line J774. European Journal of Immunology 28:1570-1577

129. **Elsner J, Oppermann M, Czech W, Dobos G, Schopf E, Norgauer J, Kapp A** 1994 C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. European Journal of Immunology 24:518-522

130. **Belmont HM, Hopkins P, Edelson HS, Kaplan HB, Ludewig R, Weissmann G, Abramson S** 1986 Complement activation during systemic lupus erythematosus. C3a and C5a anaphylatoxins circulate during exacerbations of disease. Arthritis and Rheumatism 29:1085-1089

131. **Moxley G, Ruddy S** 1987 Elevated plasma C3 anaphylatoxin levels in rheumatoid arthritis patients. Arthritis and Rheumatism 30:1097-1104

132. **Bengtsson A, Heideman M** 1988 Anaphylatoxin formation in sepsis. Archives of Surgery 123:645-649

133. **Murakami Y, Yamamoto T, Imamichi T, Nagasawa S** 1993 Cellular responses of guinea-pig macrophages to C4a; inhibition of C3a-induced O2- generation by C4a. Immunology Letters 36:301-304

134. **Gerard NP, Gerard C** 1991 The chemotactic receptor for human C5a anaphylatoxin. Nature 349:614-617

135. **Crass T, Raffetseder U, Martin U, Grove M, Klos A, Kohl J, Bautsch W** 1996 Expression cloning of the human C3a anaphylatoxin receptor (C3a R) from differentiated U-937 cells. European Journal of Immunology 26:1944-1950

136. **Ames RS, Li Y, Sarau HM, Nuthulaganti P, Foley JJ, Ellis C, Zheng ZZ, Su K, Jurewics AJ, Hertzberg RP, Bergsma DJ, Kumar C** 1996 Molecular cloning and characterization of the human anaphylatoxin C3a receptor. Journal of Biological Chemistry 271:20231-20234

137. **Hollmann TJ, Haviland DL, Kildsgaard J, Watts K, Wetsel RA** 1998 Cloning, expression, sequence determination, and chromosome localization of the mouse complement C3a anaphylatoxin receptor gene. Molecular Immunology 35:137-148

138. **Fukuoka Y, Ember JA, Hugli TE** 1998 Molecular cloning of two isoforms of the guinea pig C3a anaphylatoxin receptor: alternative splicing in the large extracellular loop. Journal of Immunology 161:2977-2984

139. **Gasque P, Singhrao SK, Neal JW, Wang P, Sayah S, Fontaine M, Morgan BP** 1998 The receptor for complement anaphylatoxin C3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. Journal of Immunology 160:3543-3554

140. **Ischenko A, Sayah S, Patte C, Andreev S, Gasque P, Schouft MT, Vaudry H, Fontaine M** 1998 Expression of a functional anaphylatoxin C3a receptor by astrocytes. Journal of Neurochemistry 71:2487-2496

141. **Martin U, Bock D, Arseniev L, Tornetta MA, Ames RS, Bautsch W, Kohl J, Ganser A, Klos A** 1997 The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. Journal of Experimental Medicine 186:199-207

142. **Murakami Y, Imamichi T, Nagasawa S** 1993 Characterization of C3a anaphylatoxin receptor on guinea-pig macrophages. Immunology 79:633-638

143. **Mousli M, Hugli TE, Landry Y, Bronner C** 1992 A mechanism of action for anaphylatoxin C3a stimulation of mast cells. Journal of Immunology 148:2456-2461

144. **Fukuoka Y, Hugli TE** 1990 Anaphylatoxin binding and degradation by rat peritoneal mast cells. Mechanisms of degranulation and control. Journal of Immunology 145:1851-1858

145. **Ohno M, Hirata T, Enomoto M, Araki T, Ishimaru H, Takahashi TA** 2000 A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. Mol Immunol 37:407-412

146. **Cain SA, Monk PN** 2002 The orphan receptor C5L2 has high affinity binding sites for complement fragments C5a and C5a des-Arg (74). Journal of Biological Chemistry 277:7165-7169

147. **Rossner S** 2002 Obesity: the disease of the twenty-first century. Int J Obes Relat Metab Disord 26:S2-S4

148. **Guerre-Millo M** 2002 Adipose tissue hormones. Journal of Endocrinological Investigation 25:855-861

149. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities or protein utilizing the principal of protein-dye binding. Analyt Biochem 72:248-254

150. **Simon MI, Strathmann MP, Gautam N** 1991 Diversity of G proteins in signal transduction. Science 252:802-808

151. **Tang WJ, Gilman AG** 1992 Adenylyl cyclases. Cell 70:869-872

152. **Hepler JR, Gilman AG** 1992 G proteins. Trends Biochem Sci 17:383-387

153. **Lee CH, Park D, Wu D, Rhee SG, Simon MI** 1992 Members of the Gq alpha subunit gene family activate phospholipase C beta isozymes. J Biol Chem 267:16044-16047

154. **Runnels LW, Scarlata SF** 1999 Determination of the affinities between heterotrimeric G protein subunits and their phospholipase C-beta effectors. Biochemistry 38:1488-1496

155. **Rhee SG** 2001 Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem 70:281-312

156. **Nishizuka Y** 1992 Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607-614

157. **Exton JH** 1994 Phosphatidylcholine breakdown and signal transduction. Biochim Biophys Acta 1212:26-42

158. **Gustavsson L, Moehren G, Torres-Marquez ME, Benistant C, Rubin R, Hoek JB** 1994 The role of cytosolic Ca2+, protein kinase C, and protein kinase A in hormonal stimulation of phospholipase D in rat hepatocytes. J Biol Chem 269:849-859

159. **Liscovitch M, Czarny M, Fiucci G, Tang X** 2000 Phospholipase D: molecular and cell biology of a novel gene family. Biochem J 345:401-415

160. **Balsinde J, Winstead MV, Dennis EA** 2002 Phospholipase A(2) regulation of arachidonic acid mobilization. FEBS Lett 531:2-6

161. **Axelrod J** 1990 Receptor-mediated activation of phospholipase A2 and arachidonic acid release in signal transduction. Biochem Soc Trans 18:503-507

162. **Krugmann S** 1999 Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. J Biol Chem 274:17152-17158

163. **Leevers SJ, Vanhaesebroeck B, Waterfield MD** 1999 Signaling through phosphoinositide 3-kinases: the lipids take center stage. Curr Opin Cell Biol 11:219-225

164. **Carlsen J, Christiansen K, Grunnet N, Vinten J** 1999 Involvement of PI 3-kinase and activated ERK in facilitating insulin-stimulated triacylglycerol synthesis in hepatocytes. Cell Signaling 11:713-717

165. **Clarke JF, Young PW, Yonezawa K, Kasuga M, Holman GD** 1994 Inhibition of the translocation of GLUT1 and GLUT4 in 3T3-L1 cells by the phosphatidylinositol 3-kinase inhibitor, wortmannin. Biochem J 3000:631-635

166. **Vanhaesebroeck B, Waterfield MD** 1999 Signaling by distinct classes of phosphoinositide 3-kinases. Experimental Cell Research 253:239-254

167. **Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P, Hawkins PT** 1997 The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. Cell 89:105-114

168. **Toker A, Cantley LC** 1997 Signaling through the lipid products of phosphoinositide-3-OH kinase. Nature 387:673-676

169. **Yart A, Chap H, Raynal P** 20002 Phosphoinositide 3-kinases in lysophosphatidic acid signaling: regulation and cross-talk with the Ras/mitogen-activated protein kinase pathway. Biochim Biophys Acta 1582:107-111

170. **Prusty D, Park BH, Davis KE, Farmer SR** 2002 Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes. Journal of Biological Chemistry 277:46226-46232

171. **Dutil EM, Toker A, Newton AC** 1998 Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). Curr Biol 8:1366-1375

172. **Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A** 1998 Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. Curr Biol 8:1069-1077

173. **Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ** 1998 Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science 281:2042-2045

174. **Cases S, Stone SJ, Zhou P, Yen E, Tow B, Lardizabal KD, Voelker T, Farese RV Jr.** 2001 Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. J Biol Chem 276:38870-38876

175. **Farese RV, Jr., Cases S, Smith SJ** 2000 Triglyceride synthesis: insights from the cloning of diacylglycerol acyltransferase. Current Opinion in Lipidology 11:229-234

APPENDIX