IDENTIFICATION OF THE ASP RECEPTOR

David Kalant

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Dr. K. Cianflone

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_____
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ABSTRACT

IDENTIFICATION OF THE ASP RECEPTOR

David Kalant, Ph.D.
Concordia University, 2004

Acylation Stimulating Protein (ASP) is a potent stimulator of triglyceride synthesis (TGS) and glucose transport in human skin fibroblasts, muscle cells and, particularly, adipocytes. It is produced through the interaction of three proteins of the alternate complement pathway of the immune system, C3, factor B and adipsin. These three proteins are secreted from adipose tissue, and interact in plasma to produce C3a, which is then desarginated at the C-terminal to form ASP. ASP then acts back on adipose tissue in an autocrine fashion. ASP production is stimulated by dietary fat, in the form of chylomicrons, and thereby increases the efficiency of uptake of dietary fat in much the same manner as insulin increases glucose uptake. Although ASP and C3a differ by only one amino acid, ASP does not bind to the C3a receptor, and is generally thought to be inactive in the immune system. Since initial binding studies indicated the existence of a specific ASP receptor, studies were undertaken to identify the ASP receptor. The results demonstrate that C5L2, originally cloned as an orphan G protein coupled receptor, is a functional ASP receptor. C5L2 binds ASP with high affinity and can mediate the TGS stimulatory function of ASP when transfected into cells that are not normally responsive to ASP. Furthermore, interfering with the expression of C5L2 in normally responsive cells diminishes the ASP effect on TGS in these cells. C5L2 is also expressed to a high degree in adipose tissue.

Previous studies in both humans and mice have provided evidence of the physiological importance of ASP in the metabolism of dietary fat. Thus the identification of the ASP receptor furthers our knowledge of a regulatory pathway that may play a role in the development of obesity, and associated cardiovascular diseases and diabetes.

RÉSUMÉ

IDENTIFICATION DU RÉCEPTEUR ASP

David Kalant, Ph.D.
Concordia University, 2004

La protéine ASP (*acylation stimulating protein)* est un puissant stimulateur de la synthèse de triglycérides (sTG) et de transport du glucose dans les fibroblastes de peau humaine, les muscles squelettiques et particulièrement les adipocytes. Elle origine de l'interaction de trois protéines issues du système immunitaire dans la voie alternative du complément : la protéine C3, le facteur B et l'adipsine. Ces trois protéines sécrétées par le tissu adipeux interagissent dans le plasma pour former le produit C3a, puis l'ASP par le retrait de l'arginine à l'extrémité C terminale. L'ASP agit de nouveau sur le tissu adipeux selon un mode autocrine. La production de l'ASP est stimulée par les graisses de la diète provenant de chylomicrons. À l'instar de l'insuline qui accroît la captation du glucose, l'ASP augmente efficacement la captation des graisses de la diète. Bien que l'ASP ne diffère du C3a que par un seul résidu d'acide aminé, l'ASP ne lie pas le récepteur C3a et apparaît inactif dans le système immunitaire. Puisque des études de liaisons ont démontré l'existence d'un récepteur ASP spécifique, les travaux entrepris dans cette thèse visaient à identifier le récepteur ASP. Les résultats de ces travaux démontrent que C5L2, initialement identifié par clonage comme un récepteur couplé à une protéine G, est le récepteur fonctionnel de l'ASP. C5L2 lie l'ASP avec grande affinité et peut engager les fonctions sTG de l'ASP lorsque transfecté dans des cellules qui normalement ne répondent pas à

l'ASP. De plus, l'expression interférée de C5L2 dans des cellules répondant normalement à l'ASP, diminue les effets de l'ASP sur la sTG. C5L2 est aussi retrouvé hautement exprimé dans le tissu adipeux. Des études antérieures chez l'humain et la souris ont fourni des évidences sur l'importance physiologique de l'ASP dans le métabolisme des graisses de la diète. L'identification du récepteur ASP ouvre nos connaissances sur une voie de régulation pouvant avoir un rôle dans le développement de l'obésité, le diabète et les maladies cardio-vasculaires qui y sont associées.

DEDICATION

I would like to dedicate my thesis to my parents, who have always supported me in my many endeavors. My father in particular has been, and remains, instrumental in teaching me to think critically, and instilling in me a healthy (and on occasion not so) desire to question.

I also dedicate my thesis to my wife Karen, for her understanding and support during the writing of this thesis.

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I would like to thank Katherine Cianflone and Dr. Allan Sniderman for providing an intellectually stimulating and supportive environment in which to carry out my work.

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To all the others I have worked with over the years in the lab, many thanks.

And finally, to Douglas Adams for his simple but vital advice:

DON'T PANIC

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ABBREVIATIONS

βarr	β-arrestin
3T3-F442A	mouse fibroblast cell line
3T3-L1	mouse fibroblast cell line
ACS	acyl-coA synthase
Ang II	angiotensin II
apoB	apolipoprotein B
ASP	acylation stimulating protein
AT	angiotensin
BK	bradykinin
BMI	body mass index
Bt_2-cAMP	dibutyryl cyclic AMP
C/EBP	CAAT enhancer binding protein
C3a	active peptide derived from complement 3
C3aR	receptor for C3a
C4a	active peptide derived from complement 4
C5a	active peptide derived from complement 5
C5aR	receptor for C5a
CAD	coronary artery disease
CD88	identical to C5aR
CR1	complement receptor 1
CVD	cardiovascular disease
DAG	diacylglycerol
db/db	natural mutation in the leptin receptor gene
desARG	missing the C-terminal arginine
DGAT	diacylglycerol acyltransferase
DMEM	Dulbeco's modified Eagle's medium
e2	2^{nd} extracellular loop
EC	endothelial cell
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
fa/fa	genetically obese rat
$FABP_{pm}$	plasma membrane fatty acid binding protein
FACS	fluoescence activated cell scanning or sorting
FAT/CD36	fatty acid transclocase
FATP	fatty acid transport protein
FFA	free fatty acid
FIAT	fatty acid incorporation into adipose tissue
FITC	fluorescein isothiocyanate

FLUOS	fluorescein hydroysuccinamide derivative
fMLP	formyl Leu-Met-Phe peptide
G protein	heterotrimeric GTP binding protein
GAP	glyceraldehyde 3-phosphate dehydrogenase
GPAT	glycerol 3-phosphate acyltransferase
GPCR	G protein coupled receptor
GPDH	glycerolphosphate dehydrogenase
GRK	GPCR kinase
HAG-CM	hepes albumin glucose calcium magnesium buffer
HDL	high density lipoprotein
HEK293	human embryonic kidney cell line
HEK-C3aR	HEK293 stably expressing C3aR
HEK-hC5L2	HEK293 stably expressing C5L2
HNF	hepatic nuclear factor
HSF	human skin fibroblast
HSL	hormone sensitive lipase
IBMX	isobutylmethyl xanthine
IFN	interferon
IL	interleukin
IRS	insulin receptor substrate
ISRE	interferon stimulated response element
KO	knock out
LDL	low density lipoprotein
LPAAT	lysophosphatidic acid acyltransferase
LPL	lipoprotein lipase
LRP	LDL receptor related protein
MAPK	mitogen activated protein kinase
NI	non-immune
NMR	nuclear magnetic resonance
ob/ob	natural mutation in the leptin gene
OM	omental
PBS	phosphate buffered saline
PKC	protein kinas C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator activated receptor
PPH-1	phosphatidate phosphatase
PT	pertussis toxin
RA	retinoic acid
RBL	rat basophilic leukaemia cell line
RQ	respiratory quotient
RT-PCR	reverse transcriptase - polymerase chain reaction
SC	subcutaneous

SREBP	sterol regulatory element binding protein
TG	triglycerol
TGF	transforming growth factor
TGS	TG synthesis
TM	transmembrane
TNF	tumour necrosis factof
TTR	transthyretin
UCP	uncoupling protein
V-A	venous - arterial
VLDL	very low density lipoprotein

SYMBOLS for DEGENERATE NUCLEOTIDES

R = G or A
K = G or T
S = G or C
W = A or T
M = A or C
Y = T or C
D = G or A or T
V = G or A or C
B = G or T or C
H = A or T or C
N = G or A or T or C

CONTRIBUTION OF AUTHORS

Chapter two:

Magdalena Maslowska wrote the introduction, and sections on pro- and anti-adipogenic factors.

Katherine Cianflone wrote the sections on in vivo and in vitro ASP production.

I wrote the rest.

The data for the figures was provided by Thea Scantlebury, Magdalena Maslowska and Hong Wei Wang

Chapter three:

All the work detailed here was performed by myself.

Chapter four:

Stuart Cain performed the competition binding and degranulation assays on RBL cells.

Magdalena Maslowska performed some of the competition binding and TGS assays on HSF, HEK293 and 3T3-L1 cells.

I performed the transfections of HEK293 cells, including the production of the C5L2 stable transfectant. I also produced the fluorescently labeled ASP and performed the binding studies with it and with the C5L2 antibody. I performed the RT-PCR experiments.

Chapter five:

A summer student, Ratna Samanta, aided with the RT-PCR.

I isolated the RNA from the adipose tissue and performed RT-PCR. I designed and transfected the antisense oligos. I performed the antisense and the β-arrestin experiments.

CHAPTER ONE
INTRODUCTION

Throughout most of our evolution, humans have lived under conditions of alternating periods of food abundance and scarcity, which led to an overall situation of limited calories. The storage of excess energy obtained during periods of food abundance was essential for survival during periods of scarcity, and the evolution of physiological systems that increased the efficiency of energy storage led to a survival advantage. Furthermore, there was a requirement for physical activity to obtain those calories. During the last century, however, food sources in the developed world became much more reliable and higher in caloric content, and at the same time the physical activity required for survival decreased substantially. These two factors have led to the present condition of excess calorie intake and thus to the rapidly increasing development of obesity.

One of the most common measures of overweight and obesity is the body mass index (BMI): body weight in Kg / height in m^2. This measurement correlates strongly with total body fat in adults, with a BMI of 25 to 29.9 for overweight, and $\geq$ 30 for obesity. By this measurement overweight or obesity in the US has increased from 45% of the adult population in 1991 to 58% in 2001 (1). In the UK the prevalence of obesity nearly tripled between 1980 and 1989, with more than 50% of adults now either overweight or obese (2). Although the prevalence of obesity in Canada, at 51%, is somewhat lower than in the US, it is on the rise as well (3).

Obesity and Disease

Obesity is associated with several chronic diseases, including hypertension, obstructive sleep apnea and several cancers (4-6). More importantly, obesity is associated with cardiovascular disease (CVD), and type 2 diabetes. CVD accounted for over 17 million deaths worldwide in 2000, and is the leading cause of mortality (7). Obesity correlates with several risk factors for CVD, including increased numbers of small low-density lipoprotein (LDL) particles, increased serum apoB concentrations, increased triglycerides (TG), decreased high-density lipoprotein cholesterol, and increased production of the pro-inflammatory cytokines tumour necrosis factor-α and interleukin-6 (1,8).

Obesity is also associated with insulin resistance and impaired glucose tolerance, conditions that frequently lead to type 2 diabetes. Diabetes affects approximately 150 million people throughout the world, and is associated with a three fold increased risk for CVD, compared to those without diabetes. CVD accounts for 60% to 80% of the deaths of people with diabetes (8,9). The combination of a glycemic disorder with at least two of the following: obesity, high TG, or low HDL, has been termed the metabolic syndrome. The syndrome is a powerful risk factor for CVD, and the prevalence of the metabolic syndrome in the US is estimated to be approximately 24%, and increases to 44% in those over 60 years old (8).

Until very recently, obesity appeared mostly at middle age, but the combination of a high fat diet and sedentary lifestyle is now affecting younger

adults and even children with increasing frequency, contributing to the prevalence of CVD-associated disorders. Overweight in children is not as well defined as it is in adults, therefore a statistical approach - a BMI over the 95th percentile for age and gender – is used. By some estimates, 22 million children worldwide under the age of five are overweight (7). In 2000, 15% of 6-19 year olds in the US had a BMI above the 95th percentile for children studied in the 1960s (10). This trend is appearing even in very young children; 10.4% of 2-5 year olds in 2000 had a BMI above the 95th percentile of the 1960s. As it has for adults, obesity in Canadian children has also increased over the past two decades (11). The increase in childhood obesity has coincided with an increase in type 2 diabetes in children, so that in some populations it is more prevalent than type 1 diabetes (7,10). One study found a ten fold increase in type 2 diabetes in children over the period 1982 to 1994. Overweight children also manifest the same CVD-associated dyslipidemias as do adults (10).

While the increase in obesity and associated diseases appeared first in the developed world, it is now becoming a serious problem for developing countries due to rapid urbanization and the adoption of a western lifestyle (7,12). In India, for example, the frequency of diabetes in rural populations is 2.4%, compared to 11.6% in urban populations, an increase associated with a change in lifestyle (12). In China, the proportion of deaths due to CVD rose from 12% in 1957 to 36% in 1990, while the number of people consuming a high fat diet (> 30% of calories) increased two to three fold just between 1989 and 1993 (13).

Adding to the health burden caused by the adoption of the western lifestyle, many ethnic groups appear to develop components of the metabolic syndrome at a lower BMI than Caucasians. In a study comparing Filipina and Caucasian women in California, 8% of Filipinas were obese compared to 14% for Caucasians, but 34% had the metabolic syndrome *vs.* 13% for Caucasians (7). African-American children, after adjusting for BMI, have increased total, LDL and HDL cholesterol, but lower TG than Caucasian or Hispanic youth (10). In an Australian study of lean, young adults of different ethnicities, significant differences in insulin sensitivity were found, in the following order: Caucasian > Chinese > Southeast Asian (14). Although it was a small study, with the effects of fetal undernutrition perhaps playing a role, it suggests the possibility of a greater genetic predisposition to diabetes in some racial groups.

While BMI measures overall obesity, it does not take into account the distribution of body fat, and it is now apparent that omental (OM), or visceral, fat depots are more closely associated with disease than subcutaneous (SC) depots (15). The reasons for this are discussed later. While both men and women gain weight via increases in SC and OM adipose tissue, men gain more in OM tissue than do women. This difference has been strongly implicated in the higher rate of an atherogenic lipid profile in men vs. women (16).

Treating Obesity

Several studies have shown that even moderate reductions in body weight or BMI can lead to significant health improvements, and a lowered risk for

developing diabetes (17). To this end, diet and exercise are important tools in both the prevention and lowering of obesity, in both adults and children. A 10 kg weight loss is associated with a 25% reduction in total mortality, and a 40% decrease in diabetes related deaths. Even a more moderate weight loss of 5-10% can improve the risk factors for CVD (2,6). A Finnish study reported that dietary changes and moderate exercise of ≥ 30 min/day resulted in a 58% reduction in the risk of diabetes in patients with metabolic syndrome (8). Other evidence suggests that for some individuals, 30 minutes may be insufficient to prevent weight gain. The prevention of weight regain in formerly obese patients may require 60 to 90 minutes of moderate, or shorter periods of more vigorous exercise (18). Even without weight reduction, aerobic activity can improve glucose tolerance (10). When affected individuals have difficulty making lifestyle changes, or when an aid to making such changes is desired, pharmaceutical intervention is frequently helpful (1,4,8). Both cholesterol lowering and insulin sensitizing drugs have been effective in reducing the risk of developing CVD and diabetes, and drugs that inhibit absorption of dietary fat, or increase satiety can help produce and maintain weight loss.

METABOLISM OF DIETARY FAT

Because of the prevalence of obesity and its associated health risks, there is an increasing focus on fat metabolism and its regulation. Research in several areas is providing a better understanding of how the human body handles dietary fat, and should reveal why some individuals are more susceptible than others to

the deleterious consequences of our present lifestyle. These areas include i) intestinal absorption of fat; ii) the storage of fat in adipose tissue; iii) the uptake of fat by the liver and its subsequent secretion in very low density lipoprotein (VLDL) particles; iv) the conversion of VLDL to the more atherogenic LDL; v) the balance between fat and carbohydrate as fuel sources, particularly in muscle; vi) signals to and from the brain that regulate eating behaviour. The rest of this chapter will focus primarily on fat storage in adipose tissue.

Dietary fat, mostly in the form of TG, is hydrolyzed in the small intestine by pancreatic lipase to release free fatty acids (FFA) and glycerol, which can then be absorbed by intestinal mucosal cells. How the mucosal cells re-secrete the FFA depends on the length of the hydrocarbon chain. Fatty acids of eight or fewer carbons are secreted as FFA into the blood, where they are bound by albumin, and then enter the portal vein. Fatty acids of ten or more carbons, which make up more than 80% of dietary fat, are re-esterified to glycerol-3-phosphate to form TG and packaged into a chylomicron. This lipoprotein particle is composed of a core of neutral lipids including TG, surrounded by a phospholipid monolayer and associated apoproteins, including apoB. Unlike other nutrients transported across the intestinal mucosa, chylomicrons are not secreted directly into the blood, but into the lymphatic system (19). The lymphatic system drains into the general circulation in the peripheral tissues and the TG is thus directed first to tissues such as adipose and muscle rather than the liver.

Hydrolysis of TG in Peripheral Tissues

When chylomicrons reach the peripheral tissues, their first interaction is with lipoprotein lipase (LPL), which is attached to heparin sulphate proteoglycans on the surface of capillary endothelial cells (20). It has been demonstrated that each chylomicron interacts with many LPL molecules (21). LPL acts both to bind chylomicrons, through an interaction with the chylomicron – associated apolipoprotein CII (22), and to hydrolyse TG to FFA and glycerol. While the glycerol remains in the circulation and is subsequently taken up by the liver, there are two possible fates for the FFA: they can enter the interstitial fluid and be taken up by the underlying tissue, or they can leave in the venous plasma, bound to albumin.

As the core TG is being hydrolyzed and FFA released from the chylomicron, the particle decreases in size to become a chylomicron remnant, which detaches from the endothelium and re-enters the circulation. The detachment is facilitated by the release of LPL from heparin sulphate, which occurs when the FFA concentration rises in the immediate vicinity of the chylomicron (23). Some LPL may remain attached to the chylomicron remnants (23,24) and may play a role in their eventual receptor–mediated endocytosis into the liver (380).

Although chylomicron TG reaches peripheral tissues first, the liver actually takes up approximately 40% of the dietary TG through the uptake of chylomicron remnant particles. Unlike adipose tissue, the liver has a limited capacity for storing TG, and it re-secretes TG in VLDL particles. VLDL is acted on by LPL in peripheral tissues, and in the postprandial state VLDL competes with

chylomicrons for LPL. Thus the fat that peripheral tissues do not take up from chylomicrons post-prandially, can eventually be taken up from VLDL.

LPL is not synthesized by endothelial cells, but by the parenchymal cells, such as adipocytes and myocytes (25,26). It is then translocated across the endothelial cells to the vascular surface (27,28). LPL expression differs in adipose and muscle tissue, and changes occur in each depending on the nutritional state (27,29,30). During the post-prandial period, LPL secretion is up-regulated in adipose tissue and down-regulated in muscle, so that FFA are taken up preferentially by adipose tissue for storage during a time of energy surplus. The opposite is true during fasting, with suppression of LPL in adipose tissue and activation in muscle, thereby directing FFA to the tissue that requires them for oxidation. There are also differences in LPL expression in different adipose depots, particularly in women. Subcutaneous adipocytes from females express LPL to a higher degree than omental adipocytes (20), and this may be one reason for the preferential storage of FFA in subcutaneous adipose tissue in women.

Fatty Acid Transport into Cells

FFAs that are released during chylomicron TG hydrolysis follow a concentration gradient into the interstitial space, bound to albumin. There is an ongoing debate concerning the mechanism by which FFA is subsequently transported across plasma membranes. On one hand, some take the position that FFAs cross the membrane by a purely diffusive process, without the

requirement of protein mediators. Some studies have suggested that this process is rapid enough to account for all FFA transport (31). On the other hand, many investigators believe that FFA transport is mediated by specific membrane proteins (31-33). A number of proteins have been identified that up-regulate FFA transport, including fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein ($FABP_{pm}$) and several intracellular fatty acid transporters (FATP1 to FATP6). Whether these proteins directly mediate FFA transport from one membrane leaflet to the other, or as some suggest, facilitate transport by acting as FFA traps, has not been proven fully.

Six highly homologous human FATPs have been found to date, and are differentially expressed in tissues that utilize FFA. FATP1 is the major FATP in adipose tissue, and also is found in skeletal and heart muscle. FATP4 is expressed exclusively in the small intestine, and FATP6 is found predominantly in the heart, where it is the major FATP (34). While FATP1 was cloned by its ability to stimulate FFA uptake into cells (35), a subsequent study showed that it has intrinsic acyl-CoA synthetase (ACS) activity (36), and a functional association with ACS has been shown for FATP2 and FATP4 as well (37,38). The ACS activity, and the predicted single transmembrane domain of FATP1, unusual for a transport protein (between 4 and 12 transmembrane domains for hydrophilic substrates) (39,40), has led some to doubt the role of FATPs as true transporters. Rather, it is suggested that FATPs function by trapping FFA in the cell via ACS activity, thereby facilitating FFA transport.

Regulation of transporters by both hormones and environmental conditions has been documented. In adipocytes, insulin increases long chain ACS but down-regulates FATP1 expression. However this does not lead to any change in FATP1 protein, but in fact increases translocation of FATP1 from the cytosol to the plasma membrane, suggesting an increase in activity (33). Insulin also causes FAT/CD36 translocation, and may regulate expression (32). Chronic muscle contraction over 7 days induced by electrical stimulation in rodents increases FAT/CD36 expression and FA transport, while training in humans and rodents increases muscle $FABP_{pm}$ expression (32).

FAT/CD36, FABPpm and FATP1 all have been implicated in diseases such as obesity, diabetes and atherosclerosis/hyperlipidemia. Specifically, in a rat model of type 1 diabetes, FAT/CD36 expression increased in muscle and heart while $FABP_{pm}$ increased only in heart. In obese Zucker rats, FAT/CD36 translocation increased in muscle (32) and heart (41). In both models FA transport was increased concurrently in muscle and heart.

Furthermore, the FATP1 gene has been linked directly to human dyslipidemia. A common polymorphism in the 8^{th} intron of the human FATP1 gene is associated with increased postprandial lipemia, including elevated triglycerides, and smaller LDL particles (42). Since FATP1 is expressed at higher levels in peripheral tissues than in liver, a reduction in FATP1 activity might therefore lead to lower uptake of FFA in peripheral tissues, and subsequent increased uptake in liver.

Although the exact mechanism of FFA uptake remains uncertain, regulation of FFA transport into cells is clearly important in the metabolism of dietary fat, and therefore in the manifestation of obesity-related diseases.

Triglyceride Synthesis

Once inside the cell, FFA is converted to fatty acyl CoA by ACS, and either oxidized or esterified to glycerol 3-phosphate to form TG for storage. Because adipocytes have very little glycerol kinase activity (43,44), the glycerol released during the hydrolysis of chylomicron TG cannot act as a substrate for the synthesis of adipocyte TG. The glycerol 3-phosphate that forms the backbone of TG must therefore be derived from glucose, whose uptake is increased postprandially by the insulin-stimulated translocation of GLUT4 to the plasma membrane.

The steps involved in the conversion of fatty acyl CoA to TG are: i) esterification of a FFA to glycerol 3-phosphate at C-1 by glycerol 3-phosphate acyltransferase (GPAT) to form monoacylglycerol 3-phosphate [lysophosphatidic acid]; ii) esterification at C-2 by lysophosphatidic acid acyltransferase (LPAAT), to form phosphatidic acid; iii) dephosphorylation of phosphatidic acid by phosphatidate phosphatase (PPH-1), to form diacylglycerol (DAG); and iv) esterification at C-3 by diacylglycerol acyltransferase (DGAT) to form TG. All the enzymes are integral membrane proteins except PPH-1, which is an amphipathic, peripheral membrane protein. ACS, GPAT and LPAAT are found in

both endoplasmic reticulum (ER) and outer mitochondrial membranes (45-48), whereas PPH-1 and DGAT are found only on the ER membrane (49).

The five cloned isoforms of ACS each have wide tissue distribution, but ACS1, ACS4 and ACS5 are prominent in liver and adipose tissue, and ACS5 in intestine (50-52). ACS activity increases 100-fold when 3T3-L1 preadipocytes differentiate to adipocytes (53), due to the presence of ACS1 mRNA only in adipocytes (52), suggesting an association of ACS1 with TG synthesis. Activation of peroxisome proliferator-activated receptor gamma (PPARγ) induces ACS1 in adipose and skeletal muscle, but not liver or heart (54), concordant with its stimulation of adipocyte differentiation (55). PPARα up-regulates ACS1 mRNA directly (56,57), but may down-regulate other enzymes of the TG synthesis pathway, thereby channeling FFA towards oxidation (58). As indicated in the previous section, there also may be proteins with ACS activity on the plasma membrane.

There are two isoenzymes of GPAT, one in the ER and one in mitochondria (59). Microsomal and mitochondrial GPAT activity are equal in the liver, but the microsomal activity is approximately 10-fold higher than mitochondrial activity in other tissues (59). In differentiating 3T3-L1 cells, microsomal activity increases 70-fold (60), while mitochondrial mRNA and activity increase about 10-fold (61), suggesting both are related to enhanced TG synthesis. Hypothyroidism induces microsomal, while reducing mitochondrial GPAT activity in rat adipocytes (62), suggesting that thyroid hormone differentially regulates both microsomal and mitochondrial GPAT. Mitochondrial GPAT mRNA is upregulated by the

transcription factor, sterol regulatory element binding protein (SREBP) (63). As SREBP mRNA expression is stimulated by insulin (64), this may be one of the several ways that insulin increases TG synthesis. Conversely, leptin reduces mitochondrial GPAT mRNA expression in adipose tissue, consistent with its role in upregulating energy expenditure (65). There is also evidence of acute GPAT activation via dephosphorylation (66).

Although five members of the LPAAT family have been discovered in humans (67), the two most studied are LPAATα, expressed in all tissues, particularly in muscle, and LPAATβ, found primarily in heart, liver and pancreas (68). Most LPAAT activity is in the ER, although some is present in mitochondria (48). LPAAT activity increases 59-fold during 3T3 differentiation (53). It is unclear whether LPAAT is activated or inactivated by phosphorylation (69).

It has been suggested that PPH-1 may be an important regulatory enzyme (69), because while its activity is relatively low compared to other TG synthetic enzymes, diet and hormone-induced changes are more dramatic (49). FFA causes PPH-1 to translocate from the cytosol to the ER membrane, and activity increases concurrently (49). Insulin restores diminished PPH-1 activity in the adipose tissue of diabetic rats (70), while PPH-1 activity increases in adipose tissue of obese rats (71). On the other hand, glucagon causes translocation of PPH-1 to the cytosol, where it is inactive (49).

As DAG is also a substrate for phospholipid synthesis and involved in intracellular signaling, DGAT is the only enzyme specific for TG synthesis. It is therefore of particular interest as a regulatory step in TG synthesis. DGAT has

the highest activity in tissues specializing in TG synthesis, including adipose, small intestine and liver (59). While DGAT mRNA increases 8-fold during 3T3-L1 differentiation (72), DGAT activity increases 60-fold (53), suggesting post-transcriptional or post-translational regulation. Down-regulation of DGAT through phosphorylation was suggested by the results of a study indicating the association of a tyrosine kinase with inactivation of adipose DGAT (66). There are conflicting reports on the inhibition of hepatic DGAT by glucagon or cAMP, however cAMP activates DGAT in hamster fibroblasts (73). Although some studies have shown that TG synthesis is sensitive to changes in DGAT activity, more studies are required to elucidate all the regulators of DGAT.

The recent cloning of two DGAT genes should aid in resolving these conflicting results (72,74). DGAT 1 and DGAT2 belong to independent gene families (75,76), yet share similar specific activities (74). The three tissues in which TG is primarily synthesized, white adipose tissue, liver and small intestine, show differential expression of the two DGATs (77). DGAT1 is more highly expressed in adipose and intestine than in liver, while DGAT2 is more abundant in adipose and liver than in other tissues. DGAT1 knockout mice can still synthesize TG, presumably due to the presence of DGAT2, however they are leaner than wild type mice, show increased energy expenditure and are resistant to diet-induced obesity (78). In contrast, genetically obese rats (*fa/fa*) show an increase in DGAT mRNA in adipose tissue (71), consistent with the function of this tissue in storing excess energy.

Although the active site of DGAT, and the other TG synthetic enzymes, was initially localized to the cytosolic surface of the ER, there is some evidence for a second isoform on the lumenal side (79,80). A lumenal DGAT would presumably be required for the synthesis of TG destined for secretion in lipoprotein particles from intestinal cells and hepatocytes.

Triglyceride Hydrolysis

Skeletal and heart muscle rely on FFA oxidation to supply much of their energy needs, and during fasting periods TG stores must be used to supply these FFAs. However, most tissues have a limited capacity to store TG, so the much larger stores of TG in adipose tissue must be mobilized to supply the necessary energy. FFAs that are stored in the post-prandial period, are released during fasting, in both adipose tissue and muscle, through the hydrolysis of TG by hormone sensitive lipase (HSL). HSL is inhibited by insulin (81), so during the post-prandial period when insulin levels are high, tissues store FFAs rather than release them. When insulin returns to basal levels during fasting, the inhibition on HSL is removed. HSL is stimulated by epinephrine and glucagon, through a cAMP pathway that leads to phosphorylation of HSL.

ACYLATION STIMULATING PROTEIN

If dietary FFAs are not converted efficiently to TG for storage in adipose tissue, what would be the consequences? Presumably FFA transport into the cell

would be diminished, resulting in an increased FFA concentration in the interstial space and thus in the microvasculature of the adipose tissue. This should result in inhibition of LPL and premature release of chylomicron remnants to the circulation, leading to increased TG uptake into, and VLDL production from the liver. The ultimate consequence would be higher LDL (therefore higher apoB) and an increased risk for atherosclerosis and CVD. Based on the hypothesis that inefficient FFA storage was responsible for hyperapoB, it was postulated that there may be a factor in plasma that would up-regulate the efficiency of FFA uptake and storage postprandially, in much the same way insulin does for glucose. This factor, or part of its signaling system might be defective in a subset of hyperapoB patients.

Fifteen yeas ago, a protein fraction of human plasma was found to increase TG synthesis in human skin fibroblasts (HSF) (82), and was named acylation stimulating protein (ASP). ASP increases TG but not phospholipid synthesis, suggesting a direct effect on DGAT (83). When purified, ASP was determined to have a molecular weight of 8932 Da and a pI of 9.1. It is identical to C3a desArg (84), a supposedly non-functional metabolite of the anaphylatoxin C3a. C3a is in turn derived from C3, a component of the alternative complement cascade of the immune system. ASP is not secreted directly into plasma, but is produced through the interaction of C3 and two other proteins, factors B and adipsin, all secreted by adipocytes (85-88). It should be noted that although ASP does not have any activity in the immune system (89), C3a has similar activities to ASP on fat metabolism (90).

ASP increases TG synthesis in adipocytes to an even greater extent than in HSF (91), and it also stimulates glucose transport in adipocytes and myotubes. ASP also lowers HSL activity (81), and this combined with the increased TG synthesis and glucose uptake, lead to a net increase in FFA uptake and storage in adipose tissue. These effects are independent of, and additive to those of insulin (92). Furthermore, ASP can substitute for insulin as a stimulator of differentiation of 3T3 preadipocytes to mature adipocytes (Figure 2.4). ASP production and action will be reviewed in more detail in the next chapter.

The physiological relevance of ASP to FFA storage and lipid metabolism has been investigated via three strategies; *in vivo* human studies, cells and membranes obtained from human subjects, and ASP knockout mice. The results of this research consistently point to an important role for ASP in the efficient storage of dietary fat.

ASP in *in vivo* Human Studies

In people of normal weight (BMI < 25), plasma ASP concentrations are 21 nM on average (93). In overweight (BMI = 26-30) and obese (BMI > 30) people, ASP concentrations are significantly higher, approximately 115 nM (93). These increased levels may simply be due to the increased amount of adipose tissue, ASP being derived from adipocytes. There could thus be a feed-forward mechanism in effect: the more adipose tissue, the more ASP, and the more ASP, the greater the efficiency of fat storage and conversion of pre-adipocytes to mature adipocytes.

The finding that obese people had significantly higher plasma ASP than normal weight individuals led to the investigation of the response of ASP to weight loss. Ten obese, but otherwise healthy subjects, underwent a total fast for four weeks (94). FFA levels, already higher than control subjects before the fast, rose significantly during the fast, as expected when TG stores in adipose tissue are hydrolyzed to supply fuel for other tissues. TG and apoB levels, also higher than control before, decreased to the level of control during the fast. Although there was a significant reduction in BMI during the fast it remained well above that of the control subjects. In contrast, ASP levels, which were double those of the controls at the start of the fast, declined to control levels by the end of four weeks. This indicates that the increased ASP levels seen in obese subjects are not due solely to the increased amount of adipose tissue.

Since ASP acts predominantly on dietary fat, it was of interest to determine whether ASP production is stimulated after consuming fat, as insulin is when carbohydrates are ingested. When ASP in the general circulation was measured after an oral fat load, no increase in ASP was detected (95-97). However, in subsequent fat load studies, the venous – arterial (V-A) gradient across adipose tissue was determined by sampling over 6 hours from an arterialized hand vein, and a vein specifically draining a SC adipose tissue bed. A 2-fold increase in the V-A gradient for ASP, followed by a return to baseline, was seen between 3 and 6 hours after a high fat meal, following a rise in total plasma TG (97,98) and chylomicron TG (97). The increase in ASP release from the adipose tissue bed not only confirmed that ASP is produced in adipose tissue, but also suggested

that its production is indeed stimulated by dietary fat. The mechanism of this stimulation is discussed further in the following chapter. The reason an increase in ASP was not seen in the general circulation after an oral fat load was probably due to dilution of the blood from adipose tissue into the general circulation. The discrepancy between adipose tissue and general circulation ASP supports the role of ASP as primarily an autocrine hormone.

A negative V-A difference in TG, indicating removal of TG by adipose tissue, and an increase in calculated FFA incorporation into adipose tissue (FIAT) during the 3 to 6 hour period indicated a close temporal relationship with ASP generation. Thus the initial rise in TG may trigger the increase in adipose tissue ASP, which stimulates the efficient uptake and storage of FFA.

Differences in the above parameters were observed between lean and obese women (98). FIAT was significantly higher in the obese than the lean, suggesting a more effective storage of FFA. Possibly due to the smaller sample size and thus higher variance, the change in the V-A gradient for ASP during the post-prandial period was not significant, as it had been in the first study. However the V-A gradient for ASP was higher in the obese during the first 90 minutes, and the increase in ASP release appeared to occur sooner than in the lean. Fasting insulin was not different between the two groups, however it rose significantly higher in the obese in the first 90 minutes. Both insulin and ASP could therefore account for the more effective uptake of FFA in the obese.

The above data suggest that obese people have a greater tendency to store fat, but whether they are predisposed to be obese is a subject of debate. In a

study using stable isotope-labeled oleate, the plasma clearance of dietary FFA in women who had undergone successful gastric reduction surgery as therapy for morbid obesity was compared with that in women who had never been obese (99). Clearance of exogenous FFA was much more rapid in the post-obese women, yet oxidation was the same in the two groups, suggesting greater storage of exogenous FFA in the post-obese. The post-obese also had lower ASP, suggesting a greater ASP sensitivity. There are other reports suggesting a predisposition to obesity (100-102), however there are also reports indicating no metabolic differences between those who had been obese and those who never were (103,104).

Studies With Human Cells and Membranes

HSF isolated from subjects with high plasma levels of both apoB and ASP have been compared with those isolated from subjects with normal ASP and either normal or high apoB (105). When ASP stimulation of TG synthesis was measured, there was a significantly lower rate in the high apoB/high ASP fibroblasts than in those from normal ASP/high apoB or normal ASP/normal apoB subjects. A similar pattern was observed for ASP-stimulated glucose transport, while there was no difference in the effect of insulin on either TG synthesis or glucose transport. The reduced ASP effect corresponded to an approximately 50% decrease in ^{125}I-ASP binding to the cell surface of the high apoB/high ASP fibroblasts, suggesting a lowered expression of an ASP receptor in these subjects. This finding supports the hypothesis that a defect in the ASP pathway

may be one cause for hyperapoB. If the clearance of FFA from plasma into adipose tissue is delayed, the stimulus on ASP production would be prolonged, leading to the high ASP levels observed in these subjects. Thus the combination of high apoB and high ASP might define an ASP resistance, in the same way that high glucose and high insulin defines insulin resistance.

As mentioned earlier, increases in omental (OM) adipose tissue are more closely related to CVD and diabetes than subcutaneous (SC) adipose tissue. The basal rate of lipolysis is lower in SC than in OM adipose tissue (106), while the rate of TG synthesis is higher (106,107). Thus the balance between TG synthesis and lipolysis favours more efficient storage in SC than in OM tissue. This balance is affected by a number of hormones, including insulin, epinephrine (108), testosterone (109), leptin and tumour necrosis factor-α (110). To determine what role ASP might play in this relationship, the binding of ^{125}I-ASP to plasma membranes prepared from human adipose tissues was measured (111). Both total binding and affinity of ^{125}I-ASP was consistently higher in SC tissue than in OM, in both men and women, suggesting that in response to ASP, SC tissue would increase their uptake and storage of FFA to a greater degree than OM tissue. These results are consistent with the general view of SC tissue being more efficient than OM at storing fat. The difference in total binding between SC and OM was more pronounced in obese women compared to lean women, but no difference was seen in men. Similar differences between SC and OM have been reported for insulin binding and affinity (112).

ASP Function In Mice

In order to study more rigorously the effect of ASP *in vivo*, several studies with rodents have been carried out, both in animals injected with ASP and in ASP deficient mice. When C57Bl/6 mice were given an oral fat load, both TG and glucose clearance were accelerated with intraperitoneal injection of ASP, compared to mice without ASP (113). A similar result was observed in two obese mice models, leptin deficient *ob/ob* which is obese but normal for TG, and leptin receptor deficient *db/db* which is obese, hyperTG and diabetic (114). In both models ASP injection enhanced TG clearance after a fat load, with the greatest effect seen in the *db/db* mice. These data provided the first evidence of the physiological importance of ASP in TG and glucose metabolism.

C3 knockout (KO) mice have been produced to study this protein in the context of the immune system (115). Since C3 is the parent protein of ASP, these mice are obligately lacking ASP (and will therefore be referred to as ASP KO), and so can be studied as a model of ASP deficiency. When an oral fat load was fed to the mice, the TG clearance from plasma was significantly delayed in the ASP KO mice as compared to the wild type (116-118). The delay was more pronounced in males than in females. Both fasting and postprandial free FFA were higher in the KO males, suggesting impaired FFA uptake. The KO mice also manifested increased cholesterol and TG in the VLDL/LDL fraction of plasma, suggesting a higher flux of dietary FFA to the liver (116). Consistent with the observations in normal, *ob/ob* and *db/db* mice, injection of ASP improved the

TG clearance in the KO mice (116). The data lend further support to an important role for ASP in the metabolism of dietary fat.

ASP KO, compared to wild type mice, have been shown to have a 26% reduction in white adipose tissue mass in males (117), and a 59% reduction in females (119), and this reduction accounted for most of the 10% decrease in total body weight observed (119). The decrease was seen in mice on either a low fat or high fat diet. Although the total body weight and mass of adipose tissue were decreased in the KO mice, their calorie intake per gram body weight was increased by 18% relative to that of the wild type. One possible reason for the increased food consumption was a decrease in plasma leptin (117,119), an adipose-produced hormone that was found to be a satiety factor in mice (120,121). The low leptin levels might be expected from the reduction in adipose tissue mass, however the difference in leptin remained even after accounting for the changes in tissue mass. In addition, basal levels of glucose in male KO mice (117), and glucose and insulin in female KO mice (119), were moderately but significantly lower than in the wild type mice, suggesting improved insulin sensitivity.

Although the decreases in adipose tissue, body weight and leptin were greater in the female KO mice than in the male KO mice, the FFA increases were larger in the male. This corresponded to an increase in postprandial lipemia in the males, a phenomenon that was absent in the female mice. This could be due to both the larger adipose depots (122) and greater insulin sensitivity (123)

generally seen in female mice. Both would contribute to higher LPL activity, and thus enhanced TG and FFA clearance.

To assess the influence of leptin on ASP function, and whether blocking the ASP pathway could help reduce obesity, ASP KO mice were bred with *ob/ob* mice to produce a double knockout (2KO) (124). Compared to the *ob/ob* mice, there was a reduction in body weight of 14% in males and 21% in females, although both remained substantially heavier than wild type mice. After a fat load there was no difference in TG and FFA profiles between wild type and *ob/ob* mice. However both TG and FFA clearance were delayed in the 2KO mice, and this was seen not only in the males (as for ASP deficient mice), but also in females.

Obesity in *ob/ob* mice is commonly associated with increases in plasma insulin and glucose. There was no difference in fasting or postprandial glucose between *ob/ob* and 2KO mice, however fasting insulin was significantly lower in the 2KO mice, suggesting improved insulin sensitivity (124). When the wild type, *ob/ob* and 2KO were combined, there was a very close correlation between plasma insulin and body weight ($r = 0.775$, $p < 0.001$), suggesting that the reduced weight resulted in increased insulin sensitivity.

Interestingly, while food intake was substantially higher in the *ob/ob* mice compared to the wild type, it was even higher in the 2KO. Since leptin is absent in both mice, the increased food intake of the 2KO mice cannot be explained by decreased leptin. Therefore the increase in food intake in ASP KO mice mentioned above is due not simply to a decrease in leptin, as was originally

speculated. It is possible that ASP itself has a satiety effect that is lost in the ASP and 2KO mice. Although other studies have demonstrated small effects on food intake when either ASP or C3a was injected (114,125,126), the effects were neither consistent nor long-lived. It is probable that the effect of ASP deficiency on food intake is not direct.

The concurrence of reduced adipose tissue and body mass on one hand, with increased food intake on the other, leads to the question: What is happening to the excess energy? Measurements of oxygen consumption (VO_2) and CO_2 production revealed significantly higher VO_2 and CO_2 in the ASP KO mice as compared to the wild type, with little or no change in respiratory quotient (118). Respiratory quotient (RQ) is defined as CO_2 / O_2 and indicates the proportion of carbohydrate to fat oxidized. The effect was observed in both male and female mice, and indicated an increase in both fat and carbohydrate oxidation. On the other hand, *ob/ob* mice had a much lower VO_2, but higher RQ than wild type, indicating decreased energy utilization with a shift to primarily carbohydrate oxidation (124). These data are consistent with a lack of leptin, as leptin has been shown to up-regulate energy utilization (127,128) and preferentially increase fat oxidation (129). ASP deficiency in the 2KO mice restored the VO_2 almost to the level of wild type mice, but with no change in RQ (124). Thus ASP and DGAT KO mice have a similar phenotype; they both have decreased adipose tissue mass, increased energy expenditure and resistance to diet-induced obesity. Although some differences remain (food intake increased in

ASP but not DGAT KO), the similarities are suggestive of a link between ASP and DGAT.

The increase in VO_2 in the female ASP KO mice can be largely explained by the 78% increase in physical activity observed in the female, but not the male, ASP KO mice (118). In order to determine what accounted for the increased VO_2 in male KO mice, they were given a fat load containing [^{3}H]-oleate, and the incorporation of the radiolabel in individual tissues was measured. Compared to wild type, KO males had increased radiolabel in muscle and liver, but a decrease in brown adipose tissue (118). When the tissue extracts were separated into [^{3}H]-lipids and [^{3}H]-oxidation products, lipids were decreased in adipose tissues, while oxidation products were increased in muscle and liver of KO mice. The data all point to a decreased uptake of fat into adipose tissues, and an increased uptake and oxidation in muscle and liver. All the observed changes in radiolabel incorporation in the KO mice were reversed when ASP was injected at the same time that the fat load was given. The observations are consistent with the proposed role for ASP in stimulating uptake and storage of dietary fat in adipose tissue. When ASP is absent, FFA uptake is decreased in adipose tissue and redirected to muscle and liver.

In order to understand why FFA oxidation was increased without an increase in physical activity in male KO mice, the expression levels of uncoupling proteins (UCP) 1, 2 and 3, in muscle and brown and white adipose tissues were measured by RT-PCR. UCPs transport protons across the inner mitochondrial membrane, effectively uncoupling oxidation from ATP synthesis and thus

generating heat. In mice, the function of UCP1, expressed predominantly in brown adipose tissue, appears to be adaptive thermogenesis. The primary roles of the widely expressed UCP2, and the mostly skeletal muscle–expressed UCP3, are presently unclear. It is proposed that UCP2 prevents the accumulation of reactive oxygen species in mitochondria and regulates the ratio of [ATP]:[ADP], while UCP3 removes excess FFA from the mitochondrial matrix and regulates glucose metabolism in muscle (130). However, both UCP2 and UCP3 may still influence energy metabolism as a secondary effect.

Although UCP1 expression was decreased in the three tissues studied, UCP2 and UCP3 were significantly up-regulated in white adipose tissue and muscle, respectively, of male KO mice (118). The higher UCP2 and 3 expression might thus explain why energy utilization in male KO mice is higher than wild type without a change in physical activity. Male and female mice therefore exploit different mechanisms to handle the fat that is not stored in adipose tissue due to decreased TG synthesis, but they both exhibit a lean, insulin sensitive phenotype.

Conclusion

The results to date, from several experimental approaches, indicate an important physiological role for ASP in the metabolism of dietary fat. The production of ASP from adipose tissue has been demonstrated in both *in vivo* and *in vitro* studies, as has the function of ASP as a stimulant of FFA clearance and TG synthesis. Although the data are not yet conclusive, studies with human

subjects strongly suggest an association of the ASP pathway with the development of obesity, while experiments with human cells suggest a relationship to dyslipidemias, particularly hyperapoB.

The studies with mice have provided more direct evidence of the importance of ASP. Despite an increased food intake, ASP KO mice are leaner than wild type, and oxidize rather than store the excess energy. Knocking out ASP can even partially diminish the obesity of genetically predisposed mice. While it is tempting to draw conclusions from these data about the role of ASP in humans, caution is always required in this respect. While many similarities exist in the metabolism of all mammals, significant differences are present as well. A noted instance is the case of leptin. The discovery of the spontaneous mutations in mouse leptin and its receptor led to the hypothesis that these mutations were also a common cause of obesity in humans. However, a genetic lack of leptin is not common in humans. Nonetheless, all the studies have increased our understanding of the complex interplay among energy intake, storage and oxidation, and provided valuable information about the role of ASP in this regard.

The current lifestyle of physical inactivity and high calorie consumption is the underlying cause for the prevalence of obesity, diabetes and CVD, and any effective treatment of these diseases must include behavioural changes. However, such changes are frequently difficult to make, and drug therapies may be a useful aid. The ASP pathway provides a potential target for such therapies, and further investigation into the mechanisms of ASP function are therefore warranted.

CHAPTER TWO
CONTROL OF LIPOGENESIS IN ADIPOSE TISSUE AND THE ROLE OF ACYLATION STIMULATING PROTEIN

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David Kalant, Magdalena Maslowska, Thea Scantlebury, Hong Wei Wang, Katherine Cianflone

Mike Rosenbloom Laboratory for Cardiovascular Research, McGill University Health Centre, Montreal, Quebec, Canada

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Address correspondence to:

Katherine Cianflone
Cardiology, H7-30
Royal Victoria Hospital
687 Pine Avenue West
Montreal, Quebec
H3A 1A1 Canada
Telephone: (514) 842-1231 ext. 35426
Fax: (514) 843-2843
E-mail: katherine.cianflone@mcgill.ca

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Key Words: C3a desArg, adipsin, lipolysis, differentiation, leptin, TNFα

Abbreviations:

Ang II	angiotensin II
ASP	acylation stimulating protein
BMI	body mass index
C3aR	C3a receptor
EC	endothelial cell
ECM	extracellular matrix
HSF	human skin fibroblast
HSL	hormone sensitive lipase
IL	interleukin
LRP	LDL receptor related protein
OM	omental
PKC	protein kinase C
RA	retinoic acid
SC	subcutaneous
TG	triglyceride
TGS	triglyceride synthesis
TNFα	tumor necrosis factor α
TTR	transthyretin

Abstract

The complex process of differentiation and accumulation of fat stores involves sequential regulation of a number of transcription factors and culminates in increased expression of a myriad of proteins which regulate fat mass. While this process is now well defined, the role of hormones that contribute to regulation of adipose tissue differentiation and fat stores is still being elucidated. Several hormones have acute effects on lipogenesis or lipolysis, but few have been shown to directly influence differentiation of adipocytes. The aim of this paper is to review those factors with particular emphasis on acylation stimulating protein (ASP) and its potential role in both lipogenesis/adipogenesis.

ASP (C3a desArg), an adipocyte derived protein, is produced through extracellular cleavage of complement C3, which is secreted by mature adipocytes, as well as other cells. ASP production is initiated with binding of factor B (B) and cleavage by the serine protease enzyme adipsin, both also adipocyte secreted proteins. Chylomicrons, which have been shown to increase C3 and ASP, also increase the production of B and adipsin. Functional studies have demonstrated that ASP stimulates fatty acid uptake to form storage triglyceride and enhance glucose transport, in a manner similar, but additive, to insulin. This effect, however, is not mediated via the C3a receptor. In order to evaluate the role of ASP in development of adipose tissue, the effect of ASP on differentiation of 3T3-F442A cells was assessed. These cells differentiate easily with insulin and have been extensively examined to elucidate the mechanisms of

differentiation. ASP induced differentiation of 3T3-F442A cells, as demonstrated by increased triglyceride synthesis, Oil Red O staining and morphologic changes. These results are consistent with the *in vivo* data in complement C3 knockout mice (therefore functionally ASP deficient) which have reduced adipose tissue, and delayed triglyceride clearance (male mice), demonstrating a physiologic role of ASP in enhancing adipose tissue fat storage.

Introduction

Adipose tissue, long regarded as the storage organ of excess energy, is a very dynamic tissue. Lipogenesis and lipolysis are two competing processes through which adipose tissue energy stores are constantly regulated. Intracellularly, multiple enzymes are activated or inactivated to ensure that the tissue responds rapidly to physiological stimuli (131). At the same time, the dynamic nature of adipose tissue is complemented by the ability to continuously generate new fat cells (adipogenesis) (132).

The specific regulation of those processes is attained through the hormonal signaling received by the adipose cell. While it is well known that circulating hormones such as glucocorticoids, catecholamines, sex hormones and insulin affect lipogenesis, lipolysis and adipogenesis (133), there is increasing evidence that hormones secreted specifically by adipocytes may play as important a role.

The recent boom of interest in adipocyte derived factors has resulted in identification of a large group of adipocyte specific proteins. Those most recently identified include adiponectin, resistin and pref-1. They are presently subject to intensive research to understand their involvement in the regulation of adipose tissue physiology, and in particular, their potential implication in insulin resistance, obesity and diabetes (as summarized and referenced in Table 2.1). Their direct role in influencing adipogenesis and especially lipogenesis or lipolysis is presently not well defined.

Of the many factors that are now recognized as being secreted by adipose tissue, only some act as paracrine/autocrine factors and interact with receptors

on adipose tissue. Fewer still 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 (TG) synthesis and differentiation. Leptin, tumor necrosis factor α (TNFα), transforming growth factor β (TGFβ), and interleukins have all been demonstrated to be anti-adipogenic, while ASP, angiotensin II and agouti are pro-adipogenic (Table 2.2). The first part of this review will address pro- and anti-adipogenic properties of adipose tissue secreted hormones. The second part will focus mainly on the role of ASP in adipose tissue physiology.

ANTI-ADIPOGENIC FACTORS:

The discovery of leptin initiated the present cascade of research leading to the recognition of newly identified adipocyte secretagogues. Leptin effects are mainly endocrine in nature and are involved in the hypothalamic control of food intake and energy expenditure (review (134)). Since the discovery of specific leptin receptor isoforms on a variety of tissues (review (135)) including adipocytes (136), investigators have focused on understanding the peripheral effects of leptin as well as the central effects. In mice, the majority of reports suggest that leptin can increase lipolysis both *in vivo* and *in vitro* (137,138) but decrease glucose oxidation (139) and lipid synthesis independent of lipolysis (138,139). Leptin also counteracts the inhibitory effect of both insulin (140) and adenosine (141) on lipolysis. A very recent intriguing paper has suggested that the effects on lipolysis may result in enhanced substrate cycling, providing an

additional ATP wasting pathway, other than the uncoupling protein proton leak which is also stimulated by leptin (142). By contrast, leptin had no effect on glucose transport in 3T3-L1 cells and rat adipocytes (143) or on lipogenesis in rat or ovine adipocytes (144,145). Despite the presence of leptin receptors on human adipose tissue, leptin had no effect on either glucose transport or lipolysis in human cultured adipocytes (146) or in ovine adipocytes (145). Thus the peripheral effects of leptin still remain controversial.

TNFα has long been recognised as a regulator of fat cell function. The recognition that TNFα is also produced by fat cells now places this cytokine in the ever-expanding list of adipocyte autocrine factors (review (147,148)). In preadipocytes, TNFα effectively inhibits differentiation as demonstrated by reduced lipid accumulation, and decreases lipoprotein lipase (LPL), glycerolphosphate dehydrogenase (GPDH), glucose transporter 4 (Glut4), and fatty acid binding protein (147,149). In differentiated adipocytes, TNFα is a potent inhibitor of lipogenesis and a potent stimulator of lipolysis. Lipolytic stimulation is evidenced by increased glycerol release (150,151) mediated by increased hormone sensitive lipase activity (152), and decreased cyclic-nucleotide phosphodiesterase 3B and $G_{(i)}$ (153,154). Lipogenesis is just as effectively suppressed through decreased Glut4, GPDH, LPL, AcylCoA synthetase, stearoyl CoA desaturase-1 and fatty acid synthetase (152,155,156). It has been suggested that these effects are mediated solely via the TNF receptor 1 (and not TNF receptor 2) (157). Moreover, TNFα not only inhibits differentiation through disruption of mitotic clonal expansion (158), but also can

reverse adipogenesis and decrease fat droplet stores (159) causing reversion to preadipocytes (160,161) and even apoptosis (162). Finally, TNFα interferes with insulin signalling, by interfering with MAP kinase activity, insulin receptor and IRS-1 phosphorylation (148,163,164). Increased TNFα in obesity may serve to limit adipose tissue expansion by inducing insulin resistance. However, this is primarily based on animal data and only partially supported by studies in humans (review (147)). An alternate hypothesis is that, since TNFα inhibits differentiation but increases cell proliferation, it may increase adipose tissue development by increasing the number of stromal vascular cells (uncommitted preadipocytes) which may be recruited later to become adipocytes (165).

Finally, a number of interleukins have now been shown to be produced by adipocytes. These include IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10 and IL-11 (166-173). Several interleukins appear to decrease lipogenesis (through inhibition of LPL or GPDH) and reduce food intake or increase lipolysis, but by far, IL-6 appears to be the best characterised both in terms of production and of effects on adipose tissue. IL-6 is increased in obesity and diabetes (174,175) and is associated with insulin resistance (176). Lastly, TGF-β has been shown to inhibit differentiation of preadipocytes in vitro (177), although the specific mechanism of this inhibition is not fully understood.

PRO-ADIPOGENIC HORMONES:

As early as 1989, the presence of angiotensinogen (178) was demonstrated in adipocytes. Subsequently, the complete renin-angiotensin system necessary for generation of angiotensin II (Ang II) was shown to exist in adipocytes

(179,180). Ang II stimulation of TG synthesis in 3T3-L1 cells is mediated through the adipocyte release and action of prostacyclin (PGI2) (181). Development of angiotensin-deficient mice, (agt-/-), demonstrated that the mice gain less weight (21%) due to decreases in adipose tissue mass (182). Overexpressing angiotensinogen in adipose tissue of either wild type or agt-/- mice further implicates Ang II in the regulation of adipose tissue mass (183). This may be even more significant considering the increased mRNA levels of angiotensinogen in the obese state (184). While it is believed that Ang II-induced PGI2 production is mediated through AT2 receptors (185,186), somewhat contradictory results by Mallow *et al* showed that AT2 receptor is lost during 3T3-L1 differentiation (187). On the other hand, Crandall *et al* (188) proposed that AT1 receptor, which is present on both preadipocytes and adipocytes (187), may be involved in the early effects of Ang II by directing preadipocytes to begin differentiation.

It is believed that agouti and its human homologue, agouti signaling protein exert their action by interacting with one of the specific melanocortin receptors (189) found in a number of tissues as well as on adipocytes (190). In mice, dominant mutations in the agouti gene which result in ubiquitous expression of its product result in a specific phenotype characterised by yellow fur, obesity and insulin resistance (191). Transgenic mice expressing agouti specifically in adipose tissue are characterised by moderate obesity but increased sensitivity to insulin (192). Agouti increases fatty acid synthase as well as triacylglycerol content of 3T3-L1 adipocytes (193). Although in humans the agouti gene is

mainly expressed in adipose tissue (194) and is increased with differentiation (195), its role in humans is still not clear.

In the last decade, despite the increasing number of newly identified adipose tissue factors, none has been demonstrated to have greater effects in stimulating lipogenesis than acylation stimulating protein (ASP). ASP has been shown to stimulate TG synthesis and glucose transport in fibroblasts, preadipocytes and especially adipocytes in both mice and humans (reviewed in 196). Specifically, in human adipocytes, ASP stimulates TG synthesis 2 to 5 fold above baseline (91,197) and glucose transport up to 2 fold (reviewed in 196). ASP effects *in vitro* are relatively rapid, very potent and comparable to those of insulin (196). Recent evidence also suggests an effect on lipolysis (discussed below). The remainder of the review will focus on the most recent published data on ASP with additional new data on regulation of adipocyte ASP production, as well as the autocrine effects: cell interaction, signal transduction and resulting fatty acid trapping.

ACYLATION STIMULATING PROTEIN

ASP Production *in vitro*

To date, there are only a few studies that have examined ASP production in adipocytes. Murine 3T3-F442A and 3T3-L1 fibroblast cell lines which can be differentiated in culture to adipocytes are commonly used models (198,199). When Choy *et al.* measured ASP in the medium of 3T3-F442A adipocytes, they found that ASP was detected only in cells stimulated with TNFα or IL-1β (87). However, when 3T3-L1 adipocytes were used (200), ASP was generated without

the requirement of cytokines, although cytokine stimulation produced a 50% to 100% increase in ASP. It is interesting to note that the requirements for differentiation of these related cell lines are not the same. While 3T3-F442A require only insulin to induce differentiation (199), 3T3-L1 also require dexamethasone and isobutylmethylxanthine (198); perhaps these other factors also make the cell more competent to produce ASP.

In our studies with human adipocytes, ASP production was greater in cultured adipocytes as compared to pre-adipocytes and increased gradually with differentiation (91,201). Stimulating adipocytes with insulin, well known to increase fat storage as well as expression of many factors, also produced an increase in ASP (202). However, the greatest increase in ASP was seen when adipocytes were incubated with the dietary lipoproteins, chylomicrons (203). The factors responsible for the stimulation were associated with chylomicrons: transthyretin (TTR) and retinoic acid (RA) (203,204). This suggests that the active component was RA, which is recognised as an activator of RXR and RAR responsive genes in adipocytes (205).

ASP Production *in vivo*

The only studies in rodents on plasma ASP are those by Choy *et al.* (206). Although average levels were increased by 50% in obese mice, this was not statistically significant, and they suggest that levels in the systemic circulation may not be indicative of levels locally generated by adipose tissue. A positive correlation was found between ASP and plasma triglyceride (206). In humans, ASP in the general circulation was increased in obese subjects as compared to

lean (207). Recent studies have demonstrated associations of ASP (and related plasma proteins) to obesity, diabetes and CAD (see review 208).

More recently we have measured *in vivo* ASP production across a subcutaneous adipose tissue bed (97,98). Not only did fasting ASP levels correlate positively with body mass index (BMI), but so did the local adipose tissue increases in ASP during an oral fat load. Insulin levels and fatty acid uptake into adipose tissue were also correlated with ASP production. However, this local adipose tissue production of ASP did not result in increased circulating ASP. These data confirm the *in vitro* data and support the suggestion that measurements in the microenvironment of adipose tissue are more relevant to ASP production and function than those in the systemic circulation.

PRODUCTION OF ASP PRECURSOR PROTEINS

Pre-adipocytes *vs* adipocytes

ASP is not secreted directly from adipocytes, but is formed through the interaction of the proteins of the proximal alternative complement pathway, C3, B and D (also known as adipsin) (Figure 2.1). These proteins are all expressed and secreted by adipocytes, but complement components of the classical pathway, such as C2 and C5 are not (87). In a study comparing 3T3-F442A pre-adipocytes to adipocytes, C3 was expressed equally in the two cell types, adipsin was expressed only in the adipocytes, and B was expressed only when adipocytes were stimulated by TNFα (87). In contrast, this same study found that B was expressed in explanted mouse adipose tissue without the requirement of

cytokine stimulation, leading to the suggestion that the cultured cells do not behave as primary cells, or that endogenous *in vivo* factors stimulate B production. Similar results were obtained in a study that used 3T3-L1 cells, except that C3 increased substantially upon differentiation, and B, though expressed to a much higher degree upon stimulation with TNFα or IL-1β, was also expressed without cytokine stimulation (200). This was likely the reason for the previously mentioned differences in ASP production from these cell lines. We have measured the expression of B, C3 and adipsin in human adipocytes. As seen with ASP, all three proteins were expressed in pre-adipocytes and the mRNA expression was increased with differentiation of preadipocytes to adipocytes (91,201).

Effect of fat depot and age

It is suggested that omental (OM) fat is more relevant than subcutaneous (SC) to obesity-related diseases such as type-2 diabetes, cardiovascular disease and hyperlipidemias (209). Furthermore, It is becoming increasingly apparent that adipocytes from different depots have differing properties (210). In two studies of human OM and SC adipose tissue that measured the expression of genes related to ASP production, it was found that C3 was increased two-fold in OM *vs* SC tissue (211), but there was no difference in adipsin between the two depots (212). In neither study did C3 expression correlate to body mass index (BMI), however the study reporting adipsin expression looked only at tissue from non-obese subjects (BMI < 30), whereas the study that measured C3 included

subjects with a wide range of BMI (18 – 58 kg/m^2). Plasma adipsin is generally higher in obese humans (207), whereas it is decreased in several mouse models of obesity (87,206). Since B was not measured in either study, it is not known whether the increase in C3 expression would lead to an increase in ASP protein, either in obesity or in OM fat as compared to SC fat.

As humans age, total body fat tends to increase, accompanied by a change in fat distribution with larger increases occurring in OM compared to SC adipose tissue (213). C3 expression in the SC depot of middle age men (56 ± 6 yr) was twice as high as that of young men (31 ± 5 yr), even controlling for differences in body fat mass (214). It also was found that hormone sensitive lipase (HSL) activity was decreased in the older men, despite higher mRNA expression levels. The authors speculate that this apparent paradox was related to the effect of ASP on HSL activity (as discussed below).

Local response to ASP will be determined not only by production but also by receptor availability. Pertinent to that, binding studies in human adipose tissue plasma membranes in SC and OM tissue revealed a consistently greater binding and affinity of ^{125}I-ASP to SC than OM. This was particularly pronounced in SC sites from obese subjects (111).

Regulation by autocrine factors

There are many factors that have been shown to up- or down–regulate the expression of B, C3 and adipsin, most of which have been studied in cells other than adipocytes. Table 2.3 lists several cell types and how B, C3 and adipsin

protein levels are regulated in each. It should be noted that these changes are not always reflected by changes in mRNA. IL-1α and β, IL-4, IL-6 and TNFα all increase B and C3 in all cell types studied while TNFα decreases adipsin. Interestingly, these five autocrine factors are all secreted by adipocytes.

The effects of TNFα and IL-1β on complement protein expression may be explained by induction of the transcription factors C/EBPβ and C/EBPδ (as shown in mouse astrocytes), and this precedes expression of C3 (215). C/EBPβ and C/EBPδ are known to play important roles in adipocyte differentiation (216). Rosiglitazone is a thiazolidinedione used to increase insulin sensitivity in type-2 diabetic patients, and is a potent ligand of PPARγ. PPARγ is expressed predominantly in differentiated adipocytes and is known to stimulate C/EBP during differentiation (216). One study of the effects of rosiglitazone on human adipocytes did not show any stimulation of C3 (217). However in this study the incubation times were short and insulin receptor, lipoprotein lipase, HSL and PPARγ were also not upregulated by the exposure to rosiglitazone, although UCP2 was upregulated. The authors suggested that longer incubations might be required for the expression of some genes.

Regulation by endocrine factors

A number of cytokines and steroid hormones have been studied for their effects on C3, B and adipsin cellular production, some in adipocytes. All increase C3 in most of the cells studied, with the exception of glucocorticoids in endothelial cells (EC) and IFN-γ in EC and human skin fibroblasts (HSF), where

down-regulation occurs (Table 2.3). Of special note, estrogen dramatically increases C3 and several recent studies examining estrogenic or anti-estrogenic compounds view C3 changes as a key efficacy index (218-220).

Although fewer factors have been studied for their effect on B, many up-regulate B. As for C3, the exception is glucocorticoids in EC, where down-regulation of B is seen. Interestingly, TNFα and IL-1β both increase B in adipocytes, resulting in increased ASP; Peake *et al.* suggested that the rate-limiting step in cytokine-induced production of ASP in adipocytes is B synthesis (200). The situation for adipsin is more variable; down-regulation occurs as often as up-regulation (Table 2.3). Interestingly, adipsin is often regulated in the opposite direction to that of C3 and B, such as with glucocorticoids.

Since chylomicrons are the primary source of the fatty acids upon which ASP acts, we were interested to know whether chylomicrons and/or any associated factors could regulate the production of C3, B and adipsin. Previously, We have reported that chylomicrons and two associated factors, RA and TTR, increase C3 protein levels (Table 2.3) (203,204). We hypothesize that TTR mediates the transfer of the active components from chylomicrons to adipocytes, which then stimulates C3 and ASP production. Our most recent study demonstrates clearly that the C3 gene is activated by RA (204). As shown in Figure 2.2, chylomicrons also potently increase expression and secretion of both B and adipsin in human differentiated adipocytes. For B, the chylomicron effect was shown to be concentration dependent (data not shown) and the chylomicron effect was further stimulated by the addition of TTR and RA (Figure 2.2, upper panel), as shown

previously with C3 (203). The stimulation of B can also be blocked with the addition of either a polyclonal antibody to TTR or a RA antagonist (data not shown). By contrast there was no further increase in adipsin when TTR or RA were added, and the antibody or antagonist treatment had no effect suggesting that chylomicrons stimulate adipsin production through a mechanism different from C3 and B. These data, along with the above mentioned effects on ASP itself, clearly indicate a chylomicron-mediated regulation of ASP production.

Promoter analysis

Because there are several factors that have been found to alter the expression of B, C3 and adipsin, study of their promoter regions could provide more information about the pathways involved in their regulation. The promoter of the adipsin gene has not been well characterized, but some factors that regulate adipsin mRNA levels have been determined. Early work looking at the regulation of adipsin in a monosodium glutamate-induced model of obesity found that the 950 base 5' flanking region down-regulated a fusion reporter gene in obese mice, when compared to lean (221). When various lengths of the promoter were fused to the reporter gene it was discovered that only 114 bases were needed for expression of the reporter gene in adipose tissue, however the region between –687 and –743 bp of the promoter was required for down-regulation in obesity (222). This region showed higher binding of protein nuclear factors extracted from lean versus obese mice, indicating the presence of transcription factors that were less active in obese mice. The 114 base region also was identified as responsible for down-regulating the reporter gene in pre-adipocytes,

but up-regulating expression in mature adipocytes (223). These findings suggest different regions of the adipsin promoter were responsible for the up-regulation of adipsin during adipocyte differentiation, versus the down-regulation seen in rodent models of genetic and acquired obesity.

More recently, overexpressing the nuclear SREBP-1c in a transgenic mouse was shown to reduce levels of adipsin mRNA, as well as other markers of adipocyte differentiation (224). This contradicts other experiments that show overexpression of nuclear SREBP-1c promotes adipocyte differentiation (225). When C/EBPα was knocked out in mice, they died shortly after birth (226), but when C/EBPβ was substituted for C/EBPα in knock-in mice, the mice were viable and fertile (227). There were some striking changes in white adipose tissue in these mice, including marked decreases in fat accumulation and adipsin mRNA levels. These findings suggested that C/EBPα is necessary for normal adipsin expression and adipose tissue maintenance and are consistent with a role for adipsin in generating ASP, which then stimulates fat storage.

While no studies of the B promoter have been carried out in adipose tissue, the promoter has been sequenced, and specific regulating factors identified. The human and murine promoters share greater than 65% homology (228), and sites responsive to IL-1, IFN-γ and hepatocyte nuclear factor 4 (HNF-4) have been found. Responsiveness to IL-1 and IFN-γ is mediated by separate regions, which are conserved between human and mouse. The IL-1 region appears to be an enhancer element.

In murine macrophages the IFN-γ region lies between –154 and –53 bp on the B promoter, and contains both an IFN-γ activation site (GAS) and an interferon-stimulated response element (ISRE) (229). Mutagenesis of either of these sites inhibited IFN-γ induction of B, and mutagenesis of both totally abolished induction. The same authors showed that IFN-γ induced binding of phosphorylated Stat1 to GAS, and binding of interferon regulatory factor to ISRE.

HNF-4 was shown to be involved with the production of two B mRNA transcripts (2.4 and 2.7 Kb) in mouse kidney and intestine (230,231), compared to one mRNA species (2.4 kb) in other tissues. HNF-4 binds to an upstream transcription initiation site to produce the 2.7 kb mRNA. Although an HNF-4 binding site has been found in the human promoter in a similar position to that in the mouse, the longer mRNA has not been found in humans. Our own analysis of the B promoter using the Genebank analysis program (Mac Vector v6) identified, in addition, putative response elements for glucocorticoid, IL-6, thyroid hormone, and estrogen (unpublished data).

Previous analysis of the C3 promoter identified response elements for IFN-γ, IL-6, estrogen, glucocorticoid, NF-κB and thyroid hormone (232). Our analysis of the promoter revealed two putative RXR binding half-sites at –268 to –275 and –205 to –212 bp as described previously (204). Based on this, we demonstrated that RA was able to stimulate a C3 promoter-luciferase fusion reporter in transfected 3T3-L1 cells (204), and estradiol also was able to stimulate this reporter (220). We have further examined the effects of RA on B and adipsin as described below.

Regulation of the activation pathway

Not only can ASP production be regulated through synthesis and secretion of its precursor proteins (*via* differentiation or hormonal stimulation), but it can also be regulated through altering the conversion rate of the precursor C3 to ASP (Figure 2.1). ASP is generated via the alternative complement pathway when the C-terminal arginine is cleaved from C3a by circulating carboxypeptidase B. C3a in turn is generated by the cleavage of complement C3 by the convertase C3bBb, a serine protease formed when adipsin, also a serine protease, cleaves B to Bb while it is bound to C3b. C3b, in turn, is produced from the cleavage of C3: so how does this apparently closed cycle begin? It appears that C3 can convert to activated C3 (C3*), a structural and functional analog of C3b (233,234). This occurs through the spontaneous hydrolysis of an unstable thioester bond in C3. This constant "tick-over" process provides C3*Bb convertase which then can convert more C3 to C3b, thus amplifying the cycle.

It is generally accepted that activation of the pathway requires C3b to be bound to an acceptor surface, in order to stabilize the C3bBb complex (235). Although the features of a suitable surface have not been well defined and not examined at all in fat tissue, it has been shown *in vitro* that nucleophiles on acceptor surfaces covalently bind C3b (236). Carbohydrates, with their many hydroxyl groups, are thus a preferred target for binding (237). In this context, endothelial cells, adipocytes and the surrounding milieu are all potential candidate docking sites.

While normal vascular endothelial cells are resistant to complement activation (238), cytokines such as IL-1β and IFN-γ can induce the endothelium to bind C3b and activate the alternative pathway (239). The activating surface, however, was found to be the extracellular matrix (ECM) underlying the endothelial cells, and not the cells themselves: the cytokines initiate a morphological change in the cells that exposes the ECM. When purified matrix components were examined, cation-dependent binding of C3 was high on vitronectin (found in endothelial-derived ECM), moderate on fibronectin, but negative on laminin and type IV collagen.

How ASP is generated normally in the adipocyte microenvironment is unclear. Changes in collagen and matrix components occurring during differentiation (240) could potentially influence this process. As the complement proteins are secreted by adipocytes, perhaps they have more access to the ECM in the interstitial space, especially with the increased permeability of microvascular endothelium. Alternatively, perhaps the binding of chylomicrons to endothelial cells may induce the sort of morphological change that allows exposure to the ECM, or mimics that surface. Electron micrographs have shown that the cell membrane partially wraps around the chylomicrons (241), an alteration that conceivably could signal more substantial morphological changes. Adipocyte production of IL-1 or other adipocyte secretagogues (mentioned above) may also influence this process and increase ASP production.

Other adipocyte secreted proteins (factors H, I and properdin) or membrane proteins such as complement receptor 1 (CR1) and LDL receptor related protein

(LRP) are further candidates to influence ASP production. The progression of the alternative pathway can be both inhibited or enhanced by several regulators, including factors H, I, and properdin. These proteins are all expressed in adipose tissue (200,206). The soluble serum protein factor H can bind to the C3b subunit of C3bBb and cause irreversible dissociation of the C3b from Bb (242), as well as allowing the rapid degradation of C3b by a serum protease, factor I (243). The expression of factor H was shown to be significantly decreased in adipocytes compared to pre-adipocytes (206), which perhaps is another reason for the higher ASP levels secreted in the medium of adipocytes.

Properdin, a serum glycoprotein, binds weakly to surface-bound C3b, more strongly (12-fold) to C3bB and most strongly (60-fold) to C3bBb (244) thereby stabilizing the convertase. Its affinity for the three fluid-phase proteins is much weaker than for surface-bound C3b. Properdin inhibits Factor I binding to and degradation of surface-bound C3b, but not the degradation of fluid-phase C3b. These data also reinforce the role that an acceptor surface plays in complement activation. Properdin circulates as a mixture of dimer, trimer and tetramer forms (245), and it is this oligomeric structure that is thought to confer its specificity for surface-bound C3b, C3bB and C3bBb. The inactivation caused by factors H and I can be enhanced by complement receptor 1 (CR1) (246), also expressed in adipose tissue (200). CR1 is found on cell surfaces as well as in soluble form in plasma (247). The cell surface form is an integral membrane protein with several C3b binding sites. It mediates several functions including endocytosis of C3b-containing complexes (247).

Another integral membrane protein expressed in adipose tissue is the low-density lipoprotein-receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR). LRP/α_2MR is a multifunctional receptor that includes among its ligands α_2-macroglobulin, apoE-containing lipoproteins (LDL and chylomicrons) and lipoprotein lipase. In adipocytes, LRP mediates a substantial proportion (19% - 54%) of lipoprotein lipase uptake (248). Also in adipocytes, insulin increases uptake of lipoproteins *via* LRP and the action is synergistic with LPL to increase lipid uptake into adipocytes (249). Insulin increases cell surface LRP at the expense of intracellular LRP through altered endocytotic processing (250). Interestingly, a direct link between C3 binding and LRP was suggested by a recent study. LRP/α_2MR has been found to bind C3* produced by the "tick-over" process mentioned above (251). Ligand blot and uptake studies suggest that LRP can mediate binding and endocytosis of C3*. The binding was high affinity, Ca^{2+} sensitive and specific for C3* (native C3 does not bind). A single cysteine-rich binding repeat was required and RAP, the receptor-associated protein that impairs all ligand binding to LRP/α_2MR, inhibits C3* binding and clearance by LRP/α_2MR (251). Finally, cell uptake was abolished in LRP deficient cells. Alternatively, might LRP/α_2MR, through its interaction with chylomicrons, provide a scaffolding for the production of ASP?

BINDING AND ACTION OF ASP

As an autocrine hormone, we propose that the relationship between ASP and dietary fat (chylomicrons) is very similar to that between insulin and glucose. An

increase in plasma glucose concentration after a meal induces the secretion of insulin, which subsequently stimulates the uptake and storage of glucose in cells. As mentioned earlier dietary chylomicrons stimulate adipocyte ASP production within the micro environment of adipocytes, which then leads to an increase in uptake and storage of fatty acids. We have discussed the regulation of ASP production, and the focus will now turn to recent studies on the mechanism and actions of ASP.

Evidence for an ASP receptor

C3a and ASP differ by only one amino acid; nonetheless there are some very specific and significant differences in the structural requirements for binding and action of the two proteins. The main structural features of both C3a and ASP include a core of three α-helices containing three disulfide bridges, and a protruding carboxy-terminal tail (252). Neither NMR nor X-ray crystallographic studies detected any gross structural differences in the two proteins (253,254). By contrast, their biologic activities appear to be distinctive. The C3a receptor (C3aR) recently cloned (255,256) can be activated by C3a or a peptide containing only the 15 amino acids of the carboxy terminal tail of C3a. However the terminal carboxyl arginine of C3a is essential for immunologic function and neither ASP (C3a desArg) nor the corresponding 14 amino acid desArg carboxyl peptide was capable of binding or activating C3aR (257,258).

On the other hand, while cleavage of disulfide bonds in the core region does not affect C3a immunologic activity (259), the ASP lipogenic activity in adipocytes

necessitates an intact protein core. Cleavage of disulfide bridges in this region abrogates this function but not binding (260). Although the carboxy-terminal tail alone was not active for triglyceride synthesis (ASP function), it is not known whether the core is sufficient for binding or function, or whether the tail of ASP is necessary. We also found that modification of the basic residues, histidine and lysine, abrogated ASP activity (260).

Together these data suggest the existence of a receptor for ASP on responsive cells, distinct from the cloned and characterized C3aR. To examine this we carried out studies comparing the binding of ASP and C3a to various cell types. Figure 2.3 (Panel A) shows homologous competition binding of ASP and C3a to HEK293 cells stably transfected to express the C3a receptor (HEK-C3aR). As expected, C3a binds specifically to HEK-C3aR. However ASP does not bind to the C3aR expressing cells, nor can ASP compete for C3a binding. However, both ASP and C3a bind specifically to HSF, a cell responsive to ASP (Panel B).

The binding of ASP and C3a to HSF were compared by homologous and heterologous competition experiments. In HSF, in contrast to HEK-C3aR cells, ASP can compete for C3a binding (data not shown). The IC_{50} for ASP competition was 72±17 nM for ^{125}I-ASP and 376±108 nM for ^{125}I-C3a (n = 5 experiments). Similarly, C3a also could compete for ^{125}I-ASP binding with an IC_{50} of 51±4.2 nM for ^{125}I-ASP and 57±4.2 nM for ^{125}I-C3a (n = 6 experiments) (data not shown). Thus the C3aR binds only C3a. In contrast, an ASP-responsive cell binds both ligands.

These results were further supported by analysis of C3aR mRNA expression (data not shown) and membrane localization. As shown in Figure 2.3 (Panel C), fluorescently labeled polyclonal antibody to C3aR clearly shows positive staining on HEK-C3aR cells, whereas HSF are negative. No fluorescence is seen with non-immune serum. Taken together, the data suggest that ASP and C3a bind to a receptor other than the C3a receptor.

Action of ASP

Since both ASP and C3a bind to HSF to the same degree, this raises the same question regarding biologic activity measured as triglyceride synthesis (TGS). In fact, in HSF both ASP and C3a (10 μM) stimulated TGS in HSF to comparable degrees, leading to increases of 176% ± 11% and 213% ± 28% respectively above basal ($p<0.025$ *vs.* basal, n = 15). C3a might be converted to ASP through the action of cellular secreted carboxypeptidases (261). However, even in the presence of a carboxypeptidase inhibitor (Plummer's inhibitor, 10 μM), C3a maintains its stimulatory capacity (197% ± 6%, $p<0.025$ *vs.* basal) (manuscript in preparation). Although the intracellular signaling pathway of ASP has yet to be elucidated fully, we have shown that protein kinase C (PKC) is implicated in stimulation of TG synthesis in HSF (262). The diacylglycerol analog PMA (1 nM), also stimulated TGS (185% ± 19%, $p<0.025$ vs. basal), further supporting the role of PKC in ASP action as suggested previously. These results were comparable to those by insulin (206% ± 14%, $p<0.025$ vs. basal), a known stimulator of TGS. Interestingly, we previously have shown that the effect of ASP

was additive to that of insulin, implying a separate signaling pathway (196). None of these effectors (including ASP) produced significant stimulation of TGS in the other cell lines studied: HEK293, HEK-C3aR and differentiated U937 (a cell that naturally expresses C3aR), which do not bind ASP (263).

When hydrolysis of the TG in dietary lipoproteins (chylomicrons) occurs, only the fatty acids are taken up into the adipocyte, and the glycerol backbone is released into the circulation to be taken up by the liver. An alternate source of glycerol is therefore required to reform storage TG in the cell, and this is provided by glucose. ASP has been shown to increase glucose uptake into adipocytes, HSF and myocytes by stimulating the translocation of glucose transporters 1, 3 and 4 to the plasma membrane (92,264,265). Thus ASP stimulates the uptake of the two molecules necessary to enhance TGS.

The balance of TG will be influenced by the contrasting processes of TGS and lipolysis and recent research by Van Harmelan *et al.* have shown that ASP also effectively inhibits HSL activity in adipocytes (81). ASP and insulin decrease basal and norepinephrine induced fatty acid release. In contrast to TGS, protein kinase C inhibitors had no effect on ASP or insulin effects on lipolysis, while selective phosphodiesterase 3B inhibitors reversed the effects of both. Thus the intracellular signaling pathways that lead to stimulation of TGS *vs* inhibition of lipolysis may not be identical. However, inhibition of lipolysis leads to a down-regulation of both glycerol and fatty acid efflux from the cell. In fact, the combined effects of both insulin and ASP result in almost complete re-esterification of the released fatty acid. Examined in a postprandial context, since dietary

chylomicrons stimulate production of ASP and then provide the substrate for ASP action, ASP therefore increases the storage efficiency of dietary fat.

Many of the adipocyte autocrine factors described above that influence lipogenesis/lipolysis also influence adipogenesis (as described above). These include TNFα, interleukins and agouti. Thus we examined the potential of ASP on differentiation in the well-defined cell models 3T3-L1 and 3T3-F442A. Preliminary experiments suggest the responses to ASP are not limited to acute effects on TGS and lipolysis. 3T3-L1 and 3T3-F442A cells differentiated into adipocytes when exposed to insulin with IBMX and dexamethasone (3T3-L1), or insulin alone (3T3-F442A) (198,199). When ASP is substituted for the insulin in each case, differentiation also occurs in both 3T3-L1 and 3T3-F442A cells. The cells take on the characteristic morphology of fully differentiated adipocytes, with fat droplets that are visible under the microscope as stained with Oil Red O (data not shown). As shown in Figure 2.4, TGS activity increased continuously over the differentiation period in cells exposed to ASP, suggesting that ASP may also directly enhance differentiation of adipocytes.

Conclusion

Many new adipose tissue secretagogues have been identified. While the role of many of these factors has yet to be elucidated, a number have clearly demonstrated autocrine capabilities that influence fat cell development, fat

storage and mobilization. ASP is a potent stimulator of TG synthesis, and both production and action appear to be regulated in close coordination with dietary lipid. These *in vitro* effects of ASP on TGS, lipolysis and differentiation are supported by *in vivo* studies. C3 knockout mice, which are obligatorily ASP deficient since they lack the precursor to ASP, have delayed postprandial triglyceride clearance and decreased adipose tissue mass as compared to wild type mice (116,117,119). It remains to be determined in future studies whether this is due to decreased lipogenesis or decreased adipose tissue differentiation. Nonetheless, ASP appears to be an autocrine factor that can directly influence adipose tissue lipid metabolism through effects on lipogenesis, lipolysis and adipogenesis.

Table 2.1 Adipocyte secretagogues

Hormone	General Associations	Action
Adiponectin (aka: acrp / AdipoQ, apM1)	↓ in obesity, type II diabetes, CAD (266) associated with insulin sensitivity (267)	Reverses insulin resistance (268,269) ↓ LPL and lipid mass (macrophages) (268) ↑ fat oxidation (muscle and liver) (266)
Resistin / SPARC	Implicated in insulin resistance (266) ↓ by TNFα (270) and TZD (271) ↓ by insulin and high fat (272) ↑ in obesity (271), ↓ in obesity (273)	Inhibits differentiation (274)
Pref-1 / dlk	↓ with adipocyte differentiation (275,276) ↓ by glucocorticoids (275,277)	Inhibits adipocyte differentiation (275,276) Induces de-differentiation (278)

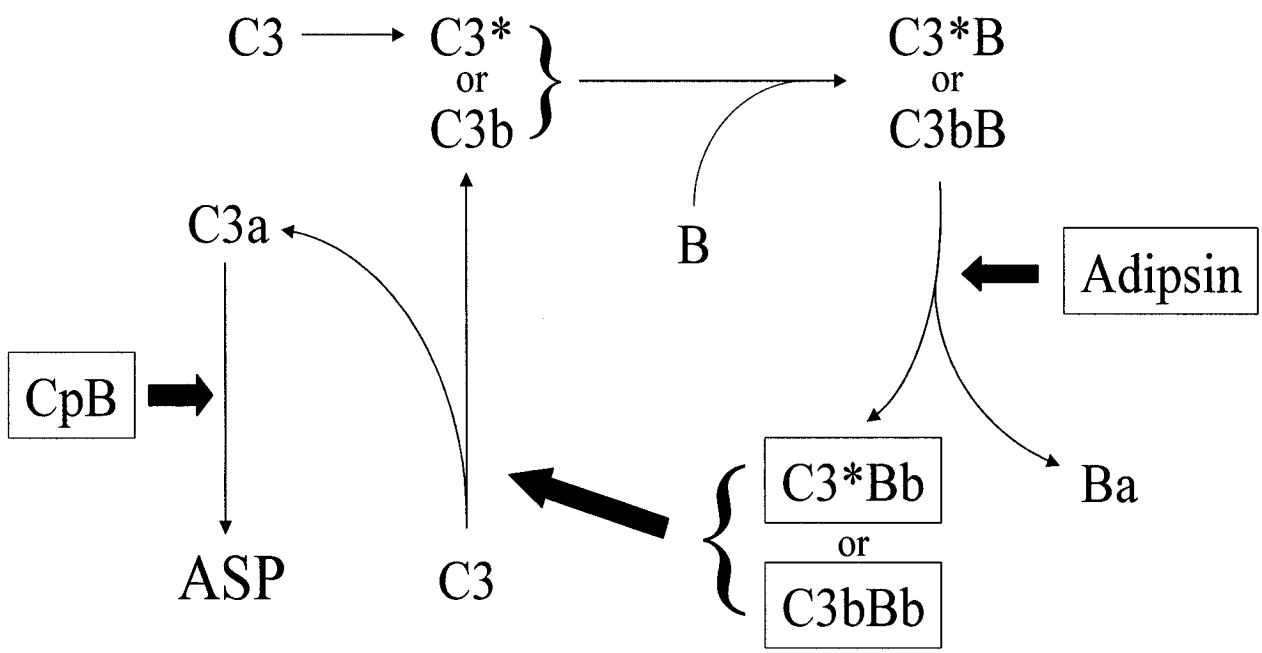
ACRP = adipocyte compliment related protein
apM = adipose most abundant gene transcript
CAD = coronary artery disease
Dlk = delta-like
LPL = lipoprotein lipase
Pref = preadipocyte factor
SPARC = secreted protein, acidic and rich in cysteine
TNF = tumour necrosis factor
TZD = thiazolidenedione

Table 2.2 Pro and anti adipogenic scretagogues

Hormone	Action	
Leptin	↑ lipolysis (138,279)↓ lipogenesis (139,148) counteracts insulin effect (140)	nc lipolysis (145,146) nc lipogenesis (144,145) nc glucose transport (146,280)
TNFα	↓ differentiation (147,149) ↓ lipogenesis (152,155,156)	↑ lipolysis (150-152) counteracts insulin effect (148,163,164)
TGFβ	↓ differentiation (177,281)	
IL	↓ lipogenesis (147)	↑ lipolysis (282)
Ang II	↑ lipogenesis (186)	↑ adipose tissue mass (182,183)
Agouti	↑ lipogenesis (193)	↑ adipose tissue mass (192)

AT = angiotensin II
IL = interleukin
NC = no change
TGF = transforming growth factor
TNF = tumour necrosis factor

Figure 2.1 Conversion of C3 to ASP:



C3 converts spontaneously to activated C3*, a C3b analog which then combines with B to form C3*B. Adipsin then cleaves the bound B to generate C3*Bb and Ba. C3*Bb is the active convertase that cleaves C3 to C3a and C3b. Carboxypeptidase B (CpB) cleaves the N-terminal arginine of C3a to produce ASP. The generated C3b can combine with B to start another cycle. The enzymes adipsin, C3*Bb (or C3bBb) convertase and CpB are indicated in boxes.

Table 2.3 Autocrine, endocrine and dietary modulation of C3, factor B and adipsin

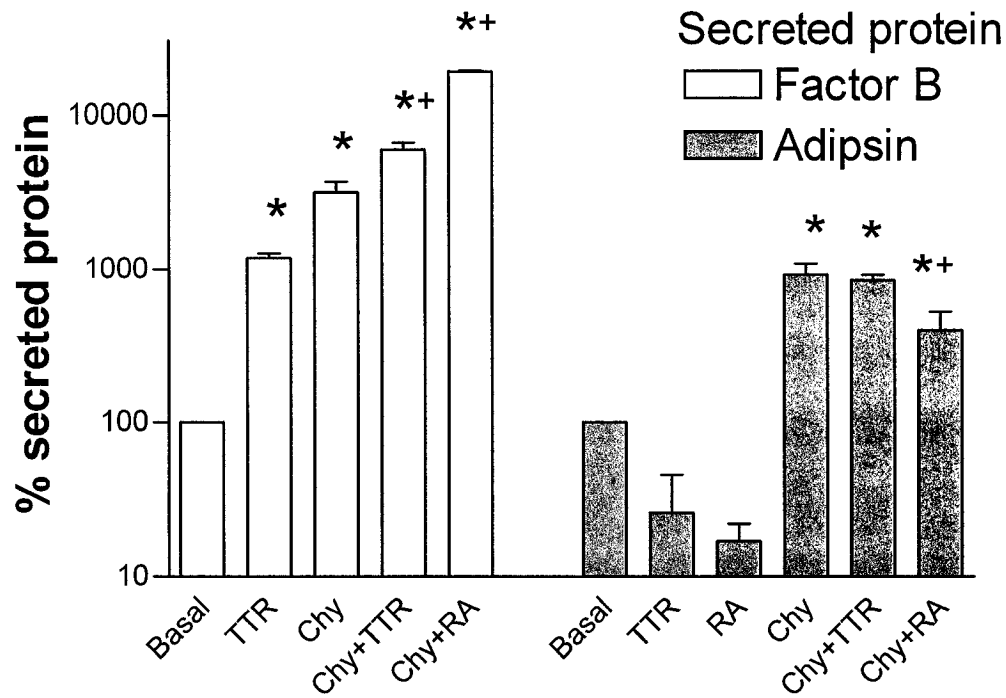
* Factors produced by adipocytes
† Factors known to be associated with lipid metabolism and insulin resistance

Adip = adipocytes
Astro = astroglioma cells
EC = endothelial cells
Epi = epithelial cells
Hepato = hepatocytes
HSF = human skin fibroblasts
IFN = interferon
IL = interleukin
Keratin = keratinocytes
Myo = myocytes
TNF = tumour necrosis factor

Table 2.3 Autocrine, endocrine and dietary modulation of C3, factor B and adipsin

	C3	B	Adipsin
IL-1α *	↑ EC (283) / Epi (284) / Keratin (285)	↑ EC (283) / Epi (284) / Keratin (285)	
IL-1β *	↑ Epi (286-288) / Myo (287)	↑ Adip (200) Epi (289) / Myo (287)	
IL-4	↑ HSF (290)	↑ HSF (290)	
IL-6 *	↑ HSF (291)	↑ HSF (291)	
TNFα *†	↑ Epi (286,289) / HSF (291) / Keratin (285)	↑ Adip (200) / HSF (290) / Mesangial (292)	↓ Adip (293)
Aldosterone	↑ Epi (294)		
Estrogen †*	↑ Epi (295)		
Glucocorticoids †	↓ EC (284) ↑ Epi (294)	↓ EC (284)	↑ Adip (296)
Insulin †	↑ Adip (202)		↑ Adip (297,298)
IFNγ	↑ Epi (299) / Hepato (300) / Keratin (285) / Myo (287) ↓ EC (301) / HSF (301)		↓ Adip (293) / Astro (302)
IL-17	↑ HSF (303)	No change HSF (303)	
Chylomicron †	↑ Adip (203,204)	↑ Adip	↑ Adip
Retinoic acid †	↑ Adip (204)	↑ Adip	↓ Adip (304) ↑ Adip (tumor) (305)
Transthyretin	↑ Adip (203,204)	↑ Adip	↓ Adip

Figure 2.2 Effect of Chylomicrons on Factor B and Adipsin Secretion and Expression: Human preadipocytes were isolated and differentiated (201), then exposed to the indicated factors alone or in combination for 6 hours: Chylomicrons (Chy 50 μg/mL TG), transthyretin (TTR 1.25 μg/mL), retinoic acid (RA 1.25 μg/mL), polyclonal antibody TTR (pAbTTR 2.5 μg/mL), retinoic acid antagonist (RAant 3.3 μM Ro41-5253). Medium was collected to measure adipsin (207) and B, which was measured by ELISA following the procedure for C3 as described (204), using Bb monoclonal and polyclonal antibodies (Quidel, San Rafael, CA) and B (Calbiochem) as standard (upper panel). mRNA was quantified by semi-quantitative RT-PCR (91) where *$p<0.05$ *vs* basal and $^{+}p<0.05$ *vs* chylomicrons (lower panel).


Secreted protein
Factor B
Adipsin
% secreted protein
10000
1000
100
10
*
*
*
*+
*+
*
*
*+
Basal
TTR
Chy
Chy+TTR
Chy+RA
Basal
TTR
RA
Chy
Chy+TTR
Chy+RA

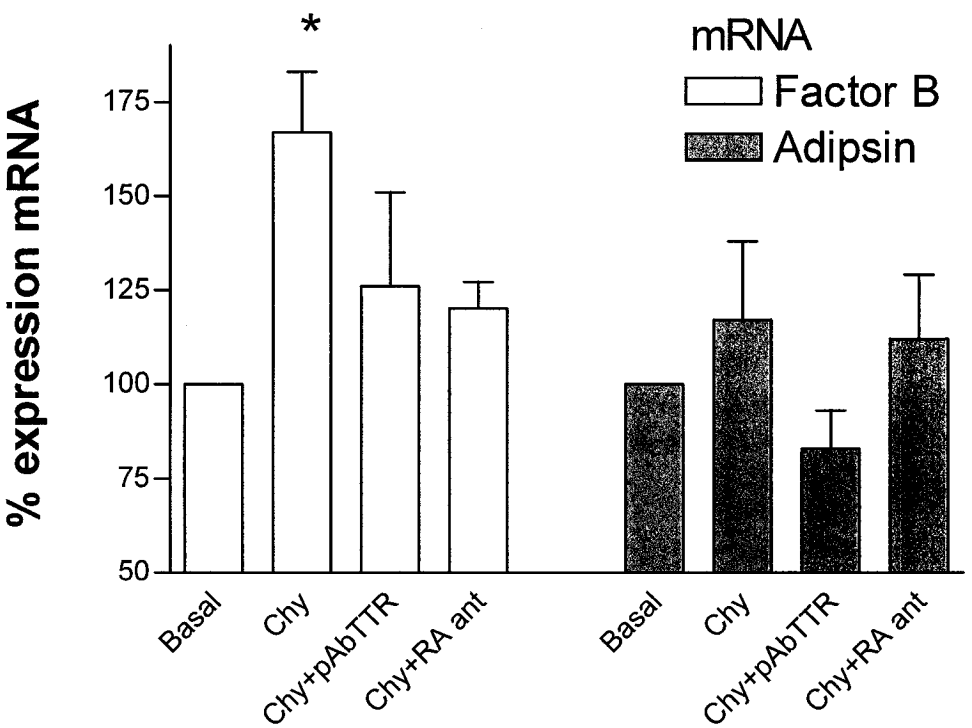
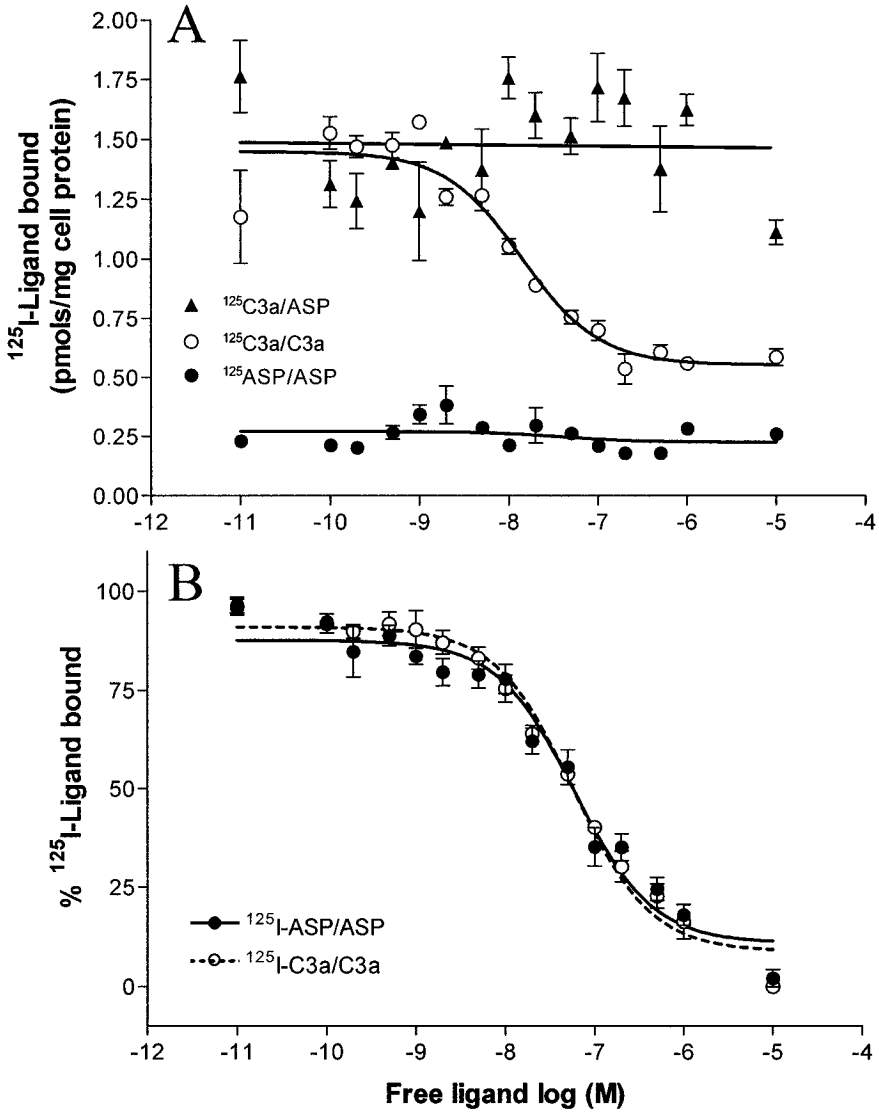

mRNA
Factor B
Adipsin
% expression mRNA
175
150
125
100
75
50
*
Basal
Chy
Chy+pAbTTR
Chy+RA ant
Basal
Chy
Chy+pAbTTR
Chy+RA ant

Figure 2.3 Competition Binding of ^{125}I-ASP and ^{125}I-C3a: C3a Plasma and ASP were purified (262) and iodinated and competition binding performed with 1 nM iodinated ligand and increasing concentrations of competitor using HEK293 cells stably transfected with human C3a receptor (Panel A) and human skin fibroblasts (HSF, Panel B) as described previously (260). Panel C: Immunofluorescent staining of HEK-C3aR and HSF cells with anti-C3a receptor polyclonal antibody and non-immune (NI) serum.


A
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
125I-Ligand bound (pmols/mg cell protein)
125C3a/ASP
125C3a/C3a
125ASP/ASP
-12
-11
-10
-9
-8
-7
-6
-5
-4
B
100
75
50
25
0
% 125I-Ligand bound
125I-ASP/ASP
125I-C3a/C3a
Free ligand log (M)

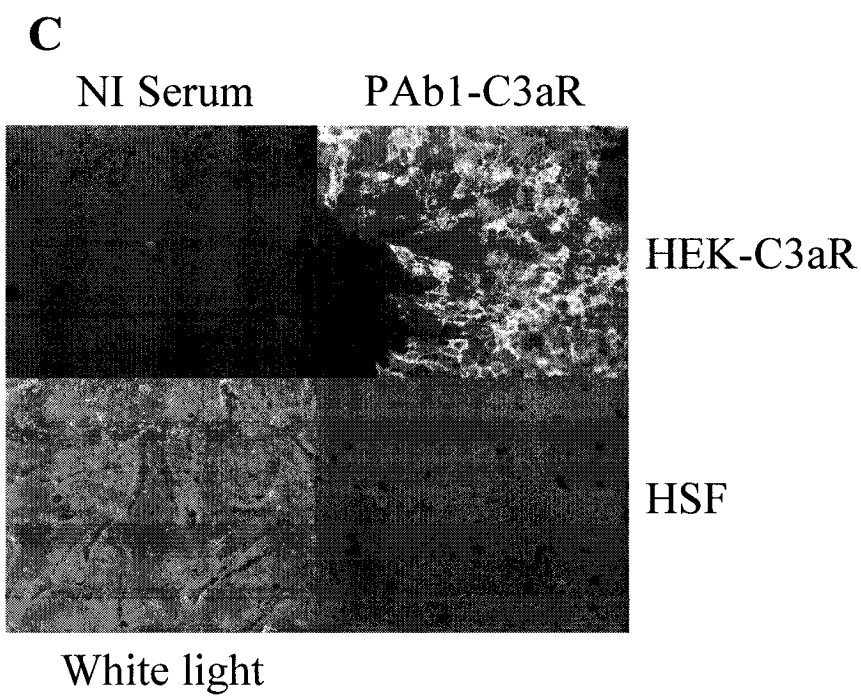
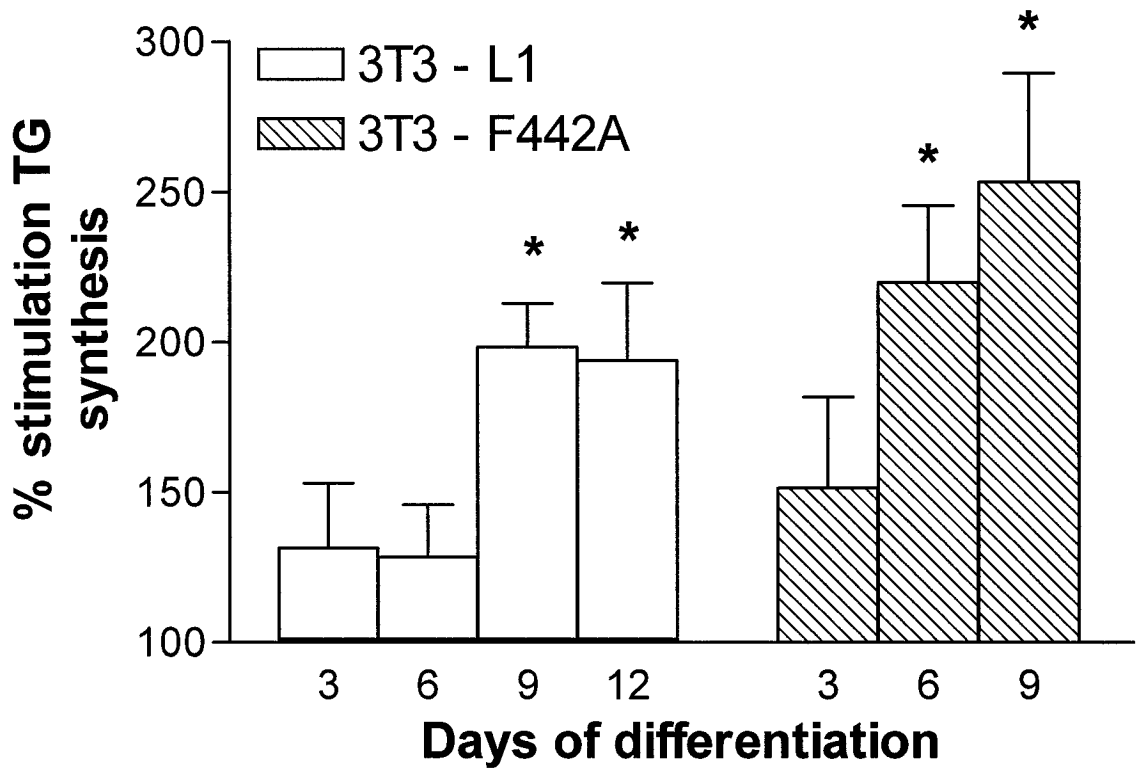

C
NI Serum
PAb1-C3aR
HEK-C3aR
HSF
White light

Figure 2.4 Pre-adipocyte differentiation with ASP



3T3-L1 and 3T3-F442A cells were differentiated as described previously (198,199). ASP (10 μM) was substituted for insulin for the differentiation period as indicated. Triglyceride synthesis (TGS) was measured as ^{3}H oleate incorporation into triglyceride for 4 hours as described (260) where *p < 0. 001 *vs* DMEM alone (for 3T3-F442A cells) or *vs* DMEM plus IBMX and DEX (for 3T3-L1 cells).

PREFACE

In the previous two chapters, the role of ASP in adipose tissue was reviewed. In order to understand the mechanism of action of ASP, and how this action might be regulated, the steps in the pathway leading from ASP to its ultimate functions must be determined. As peptides can not pass freely through plasma membranes, the first step in the pathway of a peptide hormone is the binding and activation of a cell surface receptor. Experimental evidence for the existence of an ASP receptor, distinct from that for C3a, was presented.

The direct isolation of proteins, especially membrane associated proteins, is frequently difficult. This is even more so for transmembrane proteins such as receptors. For this reason, the gene that codes for the protein of interest is usually an easier target. A cDNA library, which represents all the genes expressed in a particular tissue, is a commonly used source for cloning a particular gene. Another common approach makes use of probes based on a known gene, if there is reason to believe that the known and unknown genes have some homology. The next chapter discusses the strategies and methodologies employed in cloning the gene for the ASP receptor by screening a cDNA library with a functional assay, as well as by homology screening.

CHAPTER THREE
STRATEGIES FOR ISOLATING THE ASP RECEPTOR

Three strategies for cloning the ASP receptor are discussed in this chapter. It should be pointed out that when these studies were initiated the protein sequence of the receptor was unknown, therefore no antibodies could be produced for screening of a cDNA library. Nor was it known to which class or family the ASP receptor might belong, so screening by homology to a known receptor was not feasible. Thus it was decided to screen a cDNA library with a functional assay; the binding of labeled ligand. This method has been used for the cloning of several receptors and other proteins, from many tissues or cells, including adipocytes (35,306-310). As adipose tissue had shown the highest binding of ASP (91), it was used as the source for a cDNA library.

While the functional screening was being carried out, the C3a receptor was cloned (255,256). It then became possible to search by homology to the C3a and similar receptors. When the functional screening failed (as detailed below), two methods of homology screening were attempted: (i) screening the library with DNA probes based on the known receptors, a common cloning strategy (311-314), and (ii) using consensus sequences based on the known receptors to search nucleotide databases for orphan receptors, on the chance that the ASP receptor had already been cloned.

FUNCTIONAL SCREENING

The first strategy used to isolate the ASP receptor involved producing a cDNA library from human adipose tissue, then screening the library by binding labeled ASP.

Library Construction

Methods: Approximately 30 g of adipose tissue were obtained from a patient undergoing mammoplasty and immediately frozen in liquid nitrogen. The tissue was homogenized in batches, in Trizol (Life Technologies, Burlington, ON), with a Polytron. The tissue extract was then centrifuged at 2500g to separate the excess fat (adipose tissue is >90% fat), which was removed from the top of each tube. Total RNA was extracted from the remaining Trizol following the manufacturer's protocol and checked for integrity by formaldehyde gel electrophoresis. The mRNA was then isolated on an oligo-dT cellulose column (Life Technologies). The Copy Kit (Invitrogen, La Jolla, CA) was used to convert mRNA to cDNA, which was then ligated into the plasmid vector pcDNA1.1/Amp. The steps involved in producing the library are as follows (and Figure 3.1):

1. Producing cDNA from mRNA with AMV reverse transcriptase, using an oligo(dT) primer containing an internal Not I restriction site (5'-AACCCGGCTCGAGCGGCCGC$(T)_{18}$-3'.
2. Producing the second strand of the DNA with *E. coli* DNA polymerase I.

3. Adding, by blunt-end ligation, synthetic adapters (short double-stranded oligomers) containing a *Bst*X I overhang, then digesting with *Not* I to produce cDNA with different restriction sites on each end.
4. Size selecting the cDNA by agarose gel electrophoresis and elution of cDNA from the gel piece containing bands between 700 and 4000 bp. Smaller DNA is likely to be complementary to degraded RNA, and the vast majority of gene transcripts are less than 4000 bp.
5. Digesting the plasmid vector with *Not* I and *Bst*X I.
6. Ligating the cDNA to the plasmid. Because of the different recognition sites, the cDNA ligates unidirectionally.
7. Transforming competent *E. coli* (Top10F') by electroporation with the ligated cDNA, and growing on ampicillin. Resistance to this antibiotic is carried on the plasmid, so untransformed bacteria will not grow.
8. Isolating amplified plasmid from the bacteria. This is the cDNA library.

Results & Interpretation: Most receptors are considered to be rare messages, occuring one in about 37,000 molecules of mRNA (Life Technologies, cDNA Library Techniques – course manual). For this reason a certain minimum number of bacterial colonies is required to ensure a representative library in which rare messages can be detected. Several attempts were made to transform *E. coli,* but the transforming efficiency remained very low, so that a representative library could not be obtained. The problem appeared to be in the plasmid ligation step, but altering the reaction conditions, including the ratio of cDNA to vector (315), only marginally improved the transformation efficiency. Another cause could have

been poor ligation of the adapters to the cDNA, but altering the conditions of this reaction also did not improve the results. The restriction digest of the plasmid also was tested several times by altering the order of the two enzyme digests, purifying between digests, or using the enzymes together. During this time there were regular discussions with technicians at Invitrogen and others, and it was suggested that some tissues are believed to have unknown factors that co-purify with RNA and inhibit transformation into *E. coli.*

At this point it was decided to contract out the library construction to Stratagene, and another RNA extraction was performed from adipose tissue and checked for integrity by formaldehyde gel electrophoresis. The mRNA was isolated as before, and sent to Stratagene. The library was constructed in an engineered λ phage, ZAP Express, so that transduction, a more efficient means of getting DNA into *E. coli* than transformation, could be performed. From the amplified phage, the phagemid pBK-CMV, containing the cDNA inserts, can be excised to produce a super-coiled vector suitable for transfection and expression in mammalian cells. The results of the library construction were as follows: 1.1 x 10^6 primary plaque forming units; average insert size of 1.9 Kb; insert size range of 0.6 – 4.0 Kb.

Library Screening

Methods: A few commonly used cell lines were considered for screening of the library, including COS-7 and HEK293. These cells grow continuously in culture and are readily transfected. HEK293 had the lowest background binding

of ASP (Figure 3.2) (as was also found by Crass *et al* for the binding of C3a (255), and thus was chosen as the target for transfecting library DNA. The initial method of screening chosen was binding of fluoresently labeled ASP followed by fluorescence activated cell scanning or sorting (FACS). This method allows the detection of the fluorescence level of individual cells (Figure 3.3), or sorting of individual cells according to a chosen level of fluorescence. This method has been used to clone several other genes (35,306,308,311), and provides a rapid initial enrichment of a target gene. ASP was labeled with the fluorescein derivative FLUOS (Roche, Laval, QC) according to the manufacturer's directions. Fluorescein is the most commonly used green fluorescent label, and FLUOS is a highly reactive hydroxysuccinamide derivative that forms stable bonds with free amino groups at neutral pH. FLUOS-ASP competed out ^{125}I labeled ASP to the same degree as unlabeled ASP, indicating it would bind normally to a potential receptor (Figure 3.4).

DNA from the phagemid excision, carried out by Stratagene, was transformed into the supplied bacterium (XL1-Blue MRF') according to the instructions from Stratagene. The Wizard mini-prep kit (Promega, Madison, WI) was used to purify the plasmid from the bacteria. Transfection into HEK293 cells was carried out with Lipofectamine, a cationic lipid reagent from Life Technologies. Cells were grown to 80 to 90% confluency in T75 flasks, and 8 μg DNA per flask was transfected according to the instructions from Life Technologies. Three days later, the cells were detached with citrate-saline as recommended by the supplier of the cells (Microbix) and centrifuged for five

minutes at 600 g. After resuspending the cells in 2% bovine serum albumin in PBS, FLUOS-ASP was added to a final concentration of 50 nM and incubated for 1 hour at 37 °C, with gentle shaking. This temperature was used with the assumption that ligand would be internalised by receptor-mediated endocytosis and a higher signal would be seen compared to equilibrium binding at 4 °C. The cells were then placed on ice and sorted by FACS. The top 0.5% fluorescing cells were collected and plasmid DNA was extracted by the Hirt procedure (35,308,316). The DNA was transformed into *E. coli* for amplification, isolated with Promega's kit and used for further rounds of transfection and sorting.

At some point in the screening process the DNA must be divided into separate pools, screened and further sudivided so that individual clones can be isolated. After the third sort the transformed and amplified DNA was split into 40 pools and a portion of each pool was transfected, in duplicate, into HEK293 cells seeded into 24 well tissue culture plates. Two days later the cells were incubated with 20 nM ^{125}I labeled ASP, in buffer contaiming hepes, albumin, glucose, calcium and magnesium (HAG-CM) (255), for one hour at 37 °C. The ASP concentration was lowered compared to that used for FACS, in order to reduce background binding. This is more problematic when counting an entire pool of cells, as compared to FACS which can distinguish the fluorescence in individual cells from that in the background medium. After rinsing three times with cold HAG-CM, cell protein was dissolved in 0.1 N NaOH and counted.

Results & Interpretation: After three sorts, no increase in fluorescence above mock transfection (plasmid without insert) was seen (Figure 3.5), but this was still

a mixed population of clones, and a similar result was noted by Schaeffer during the cloning of a fatty acid transport protein (35). Several pools showed higher ASP binding than mock transfected cells (Figure 3.6), and the most consistent pool (#18) was subdivided into 40 pools and screened as above. More pools were positive, and the highest was further subdivided and screened again. At this point the binding in the duplicates was not consistent, no further increase in binding was observed (i.e., no enrichment), nor did the number of higher binding pools increase, as would be expected when a single clone is being enriched.

The process was restarted with the DNA extracted from the third sort, but this time 6-well plates were used for seeding of HEK293 cells, and the concentration of ^{125}I-ASP was lowered to 1 nM. It was reasoned that the overall signal would be increased by using more cells to internalize receptor-bound ASP, while non-specific binding to the cell surface would be decreased with the lower ASP concentration, thereby increasing the signal to noise ratio. After the first round, one pool showed a small increase in binding, and this pool was subdivided. After the third round of screening, one pool initially showed very high binding, however when it was retested twice the result could not be reproduced.

There are a number of possible reasons to explain why the library screening failed to identify an ASP receptor. The mRNA for this gene could have been degraded and not able to produce a functional protein, however, this seems unlikely since the RNA and library were checked with standard methods. Alternatively, plasmid DNA extracted from mammalian cells by the Hirt procedure sometimes becomes damaged and the inserts are lost (317). More likely, the

sensitivity of this screening method may not have been sufficient to pick up a signal. This could have been due to the sensitivity of the binding assays used, or a low level of expression in the transfected cells.

In an attempt to increase the expression level of the transfected library, the plasmid was modified. The pBK-CMV phagemid has a promoter and ATG for Lac expression upstream of where the cDNA clones are inserted. This enables blue/white screening in *E. coli*, and expression in eukaryotic cells of inserts lacking the full 5' end with the translation start site. However, this feature can lower expression of full length clones with their own ATG site in eukaryotic cells due to competition between the two translation start sites (plasmid manual). For this reason the lac promoter and ATG were excised from the original, unsorted library, and the plasmid religated. However, transformation of the religated plasmid was very inefficient, even considering that a non-supercoiled plasmid (resulting from the cutting and religating) generally transforms with lower efficiency than the supercoiled form. This appeared to confirm that the problem during the initial attempt at producing a cDNA library was in the ligation step. Whether or not removing the lac promoter and ATG would have increased the expression level is uncertain, but even if it had, the binding assay may still not have been sensitive enough. It was clear that other approaches to identifying the ASP receptor were needed.

HOMOLOGY SCREENING

While attempting the library production and screening, the cloning of the C3a receptor from the macrophage cell line U937, was published (255,256), and sequence analysis indicated a seven transmembrane, G protein coupled receptor (GPCR). Since C3a and ASP differ by only one amino acid, it was reasonable to assume that there might be a high degree of homology between the C3a and ASP receptors. However, this single amino acid difference is sufficient to prevent ASP from binding to the C3aR (255). Methods of homology screening were therefore attempted. The C3a receptor (C3aR) shows homology to the C5a receptor (C5aR), and also to the formyl Met-Leu-Phe receptor (fMLPR) and two related receptors (fML1 & fML2). Multiple alignments of these five receptors, available in Genebank from several species, were produced (using Clustal W algorithm) for two different strategies of homology cloning, which were undertaken concurrently.

Isolating Library Clones with Probes Based on Degenerate Primers

Methods: Homologous GPCRs show the greatest homology in their transmembrane (TM) regions (314,318,319). The protein sequences for the human C3aR, C5aR, fMLPR, fML1 & fML2 were aligned using the program Antheprot, and a 10 amino acid region of high homology in each of the predicted first, fourth and seventh TM regions was selected. Degenerate primers were designed to the corresponding regions in the nucleotide sequences (Figure 3.7), a forward primer for TM1 (5'-BTVBTSGGRKTSCYRGGCAATGGGCTKGTG-3'),

forward and reverse for TM4 (5'-RDAVABGAAVDHDGGHAWGSWVAKSAS HAD-3', 5'-HTDSTSMTBWSCWTDCCHDHBTTCVTBTDY-3'), and reverse for TM7 (5'-GGGKTTKADGCARCTKTTGRHRVAKGCHAR-3').

RNA from the adipose tissue of three independent subjects, and human skin fibroblasts (HSF) (sources responsive to ASP), as well as from HEK293, HEK-C3aR and U937 (sources not reponsive to ASP) (Table 4.4), were subjected to RT-PCR using the primer combinations TM1-TM4, TM4-TM7 and TM1-TM7. Two sets of primers for C3aR also were used, to ensure that any potential bands detected with the degenerate primers were not C3aR, and on the chance that these primers might amplify genes other than C3aR. PCR was carried out for 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C. As control, PCR with primers for glyceraldehyde phosphate dehydrogenase was performed under the same conditions, but for 25 cycles. Samples of each reaction were electrophoresed on a 9% polyacrylamide gel and silver-stained (BioRad, Burlington, ON). Eleven bands from the TM1-TM4 and C3aR primers were present in ASP-responsive sources (Figure 3.8), but not in ASP-nonresponsive sources (data not shown), and one band from HEK-C3aR was picked up with the TM1-TM4 primers (Figure 3.8). These twelve products were electrophoresed on an agarose gel, the bands were excised, eluted with Qiaex II kit (Qiagen, Mississauga, ON), and sent for sequencing. For sequencing of the bands detected with the degenerate primer pair, a 15 base non-degenerate oligonucleotide was made (5'-GGCAATGGGCTTG TG-3') corresponding to the 3' end of the TM1 primer.

Of the twelve bands sequenced, one matched a proteoglycan, and so was discarded. Three matched only genomic contigs and seven gave poor sequencing results. These seven were nonetheless used along with the three good sequences to produce biotinylated probes. PCR was carried out with the appropriate primers; in the case of the genomic sequences, new primers were designed to be specific for these sequences. The products were electrophoresed on an agarose gel, and portions of each band picked with a sterile Pasteur pipet to be used as a clean template in a larger reaction containing biotinylated dATP. The probes were purified with the Nucleospin kit from Clontech. To test the probes, they were spotted onto a nylon membrane and UV crosslinked according to instructions in the Photogene Nucleic Acid Detection System (Life Technologies). After blocking, the membranes were incubated with streptavidin – alkaline phosphatase conjugate, then with the detection reagent Lumi-phos 530. Spots were visualised by exposing x-ray film to the membrane.

The biotinylated probes were used with Clontech's CloneCapture kit to pull out plasmids homologous to the probes. Briefly, the steps are as follows (and Figure 3.9):

1. The probe is incubated with the plasmid library in the presence of RecA and ATPγS. RecA is a bacterial ATP-requiring enzyme that catalyses recombinations between homologous DNA. In the presence of the non-hydrolysable analogue ATPγS, a triple helical structure is formed between the probe and homologous plasmid inserts, with no recombination.

2. The RecA is digested with proteinase K, which is subsequently inactivated with phenylmethylsulphonyl fluoride. The triple helical structures are left intact.
3. Magnetic beads covalently linked to streptavidin are added, and the biotin probes bind to the streptavidin beads.
4. The tube is placed in a magnetic stand and the beads drawn to the side of the tube. Unbound plasmid is washed away.
5. The bound plasmids are freed from the probe by disrupting the triple helical structure with alkaline treatment: the probe remains bound to the magnetic bead.
6. The plasmids are purified from the supernatant, amplified in *E. coli* and used for transfection.

The plasmid pools isolated via the above method were transfected into HEK293 cells, and assessed by ^{125}I-ASP binding at 37 °C. ASP binding to 3T3 cells was used as a positive control for binding, and binding of ^{125}I-C3a to C3aR transfected cells was used as a positive control for transfection.

Results & Interpretation: Four pools showed higher binding than a mock transfection, with the counts normalized to cell protein to control for variations in cell seeding and potential loss of cells during manipulations (Figure 3.10). The four pools were further subdivided into 20 pools each. Some of these subpools showed further increases in ASP binding, but the same subpools also showed substantially lower cell protein than the negative subpools (Figure 3.11). As there was no difference among transfected cells in the counts themselves, the question

arose as to whether the apparent increase in binding was due solely to the lower cell protein.

Despite the concern that the binding might be an artifact, some of these subpools were further subdivided. However, no further increase in ASP binding was observed, nor was there any increase in the number of subpools showing higher binding. There are two possible reasons why this strategy failed. The first is that the ASP receptor is not homologous enough to the receptors used to design the degenerate primers, and/or that the probes produced from these primers were not homologous enough to bind to the ASP receptor. The second reason could be that the binding assay is still not sensitive enough to pick up a single clone among a mixture, in spite of the theoretical enrichment of a target clone. It should be pointed out that these two problems are not mutually exclusive.

Searching For Orphan Receptors

Methods: For the search for orphan receptors three consensus sequences were made from alignments of C3aR, C5aR, fMLPR, fML1 & fML2: one using the C3aR from all species, one using all the human receptors, and one using all the receptors from primates. These three sequences were used to search both nucleotide and protein databases. A list was compiled of the 123 highest scoring sequences. Thirty-three of these had putative ligands and were therefore not pursued. Researchers who had submitted several sequences to the databanks were contacted and requests were made for cDNAs in mammmalian expression

vectors. Several researchers were reluctant to share their clones, therefore some candidates were not tested. A total of 21 clones were received, 17 in plasmids, and four stably transfected into HEK293 cells. These were tested by internalization of either ^{125}I-ASP or FLUOS-ASP.

Results & Interpretation: Three received clones were not pursued when putative ligands were discovered before the orphans were tested (personal communication). Of all the orphan candidates tested (Figures 3.12, 3.13), only HD6.4, a stable transfectant, showed initial high binding (Figure 3.13), but it was not reproducible in two repeated experiments. It also showed no apparent binding to ^{125}I-C3a (Figure 3.13). Because C3a also stimulates TG synthesis, and cross competition studies indicate binding to the same site (Table 4.2), HD6.4 was ruled out as a possible ASP receptor.

Owman, using a reporter system (320), had found that C3a desArg activated one of their orphan receptors, Jaho 10, also identified as a candidate in our homology search (personal communication). When they tested ASP from our lab, they found it bound to the C5a receptor to the same degree as C5a, and to a greater degree than to Jaho 10 (personal communication). In our hands, however, subsequent testing of their plasmid containing C5aR, compared to one containing Jaho10, showed no binding of ASP (Figure 3.14). Further communications revealed that they were comparing the binding of ASP to that of a peptide fragment of C5a which binds with 1000-fold less affinity than native C5a (321,322). Their interpretation that C5aR may be an ASP receptor was therefore unconvincing.

As a positive control, ^{125}I-C3a binding to the C3a receptor was assessed using identical methodology, i.e., transient (Figure 3.13) or stable (Figure 2.3) transfection in HEK293 cells and radioligand binding. Thus the ability to transfect and detect binding to a single clone could be verified and was not in doubt. It was, therefore, concluded that the ASP receptor either (i) was not homologous to the receptors used for the database searches, (ii) was among the orphan receptors we were not given access to, or iii) equally likely, had not been cloned as of the year 2000 when the search was performed.

Return to the Original Library Screening

Methods: Since the first attempts at screening the cDNA library (1996 – 1999), new knowledge and several technical improvements had been incorporated into the overall procedures, as they became available. These are as follows:

1. The receptor for C3a had been cloned, and we now had the receptor, both in a plasmid vector and a stably transfected HEK293 cell. This provided a tool for the optimization of transfection and binding protocols with a protein that had almost identical physical properties to ASP.
2. Crass *et al.* had used a buffer (HAG-CM) that improved the sensitivity (signal-to-noise ratio) of the C3a binding assays (255), and was subsequently found to work well for ASP binding.
3. Binding on adhered cells as opposed to cells in suspension may allow interaction of the ligand with a receptor that is in a more natural

conformation. Incubation at 37 °C, to allow internalization of ligand, facilitated the use of trypsin to detach the cells for FACS, with minimal loss of signal if the ligand is cleaved from the cell surface. The initial method, citrate-saline, was less effective at detaching the cells from the tissue culture plates and caused clumping of the cells which was undesirable in using the flow cytometer. Addition of EDTA to the trypsin further improved the suspension of individual cells.

4. With the reduction in clumping, the detached cells could be fixed in suspension with paraformaldehyde (PFA), thereby stabilizing the cells before sorting or scanning.
5. Newer transfection reagents providing increased transfection efficiencies and a simpler protocol were now available. When Lipofectamine 2000 was compared to the original Lipofectamine, improvements in transfection were observed as assessed using a β-gal reporter plasmid.
6. The procedure for purifying plasmid DNA from *E. coli* with Qiagen's mini-prep kit was modified to purify plasmid from mammalian cells (communication with Qiagen), with better results than the standard Hirt method.

Based on these considerations, the original screening strategy was re-evaluated, incorporating all the new techniques and utilizing C3a-C3aR as a model system. In order to eliminate the problem of normalizing the binding to total cell protein, which was too variable, cells were screened by FACS, whereby binding per individual cell could be assessed.

After several experiments optimising FLUOS-C3a binding to HEK293 cells transiently transfected with C3aR, HEK293 cells were again transfected with the human adipose library. Approximately 5 x 10^6 cells in each of 6 T75 flasks were transfected with 100 μg of DNA. After 2 days the cells were transferred to serum-free medium, and the next day the cells were incubated with 10 nM FLUOS-ASP in HAG-CM at 37 °C for 40 minutes. Testing of various ligand concentrations with the C3a-C3aR model indicated that 10 nM gave the highest signal-to-noise ratio. After rinsing twice with HAG-CM, the cells were detached with trypsin/EDTA. The trypsin was neutralised by the addition of an equal volume of medium containing 10% serum, and the cells were centrifuged at 600g for five minutes. After fixing for five minutes with 1% PFA in PBS, the cells were centrifuged again and resusupended in 0.3% PFA in PBS.

Two rounds of sorting were carried out, with 0.2% of the top fluorescing cells being collected each time. Plasmid DNA was isolated after each sort with the modified Qiagen protocol, and used to transform *E. coli* to amplify the plasmids for another transfection. After the second sort, the plasmids were amplified in 20 separate pools, and used to transfect HEK293 cells in 6-well plates, in duplicate. DNA was transfected at 4 μg per well, which had been seeded with 1 x 10^6 cells each. After binding FLUOS-ASP as above, the cells were scanned by FACS. To quantitate the binding, a marker was set at the 95^{th} percentile on the fluorescence histogram of a mock transfection, such that 5% of the cells had a higher fluorescence than the marker. This was considered background binding.

The percentage of cells from a library transfection which fell above this marker was then determined, and divided by the background.

Results & Interpretation: In comparison to mock-transfected cells, six pools showed significantly higher binding of ASP (Figure 3.15). The two highest pools, one and nine, were further subdivided into 20 pools each. As seen in Figure 3.16, one subpool from pool nine and two subpools from pool one were higher than their respective parent pools. It appeared that with the changes in procedures, the original strategy was now producing an enrichment of an ASP-binding clone(s).

At the time that the first set of pools was being tested, studies in Monk's lab in Sheffield, England revealed that a recently cloned orphan receptor, C5L2 (325), bound recombinant C3a and C3a desArg (personal communication). In our hands, when the cDNA for C5L2 was transfected into HEK293, it bound 100% more ASP than pools one and nine, and 50% more than subpools 1A and 1F (Figure 3.17). It was decided that C5L2 was a more promising lead, and the library was put aside, with the knowledge that initial screening tests were positive, and could be pursued at a later time. Interestingly, C5L2 was independently cloned, as GPR77 (324), by O'Dowd, approximately one year after we had received other clones from him.

Figure 3.1 Library construction protocol

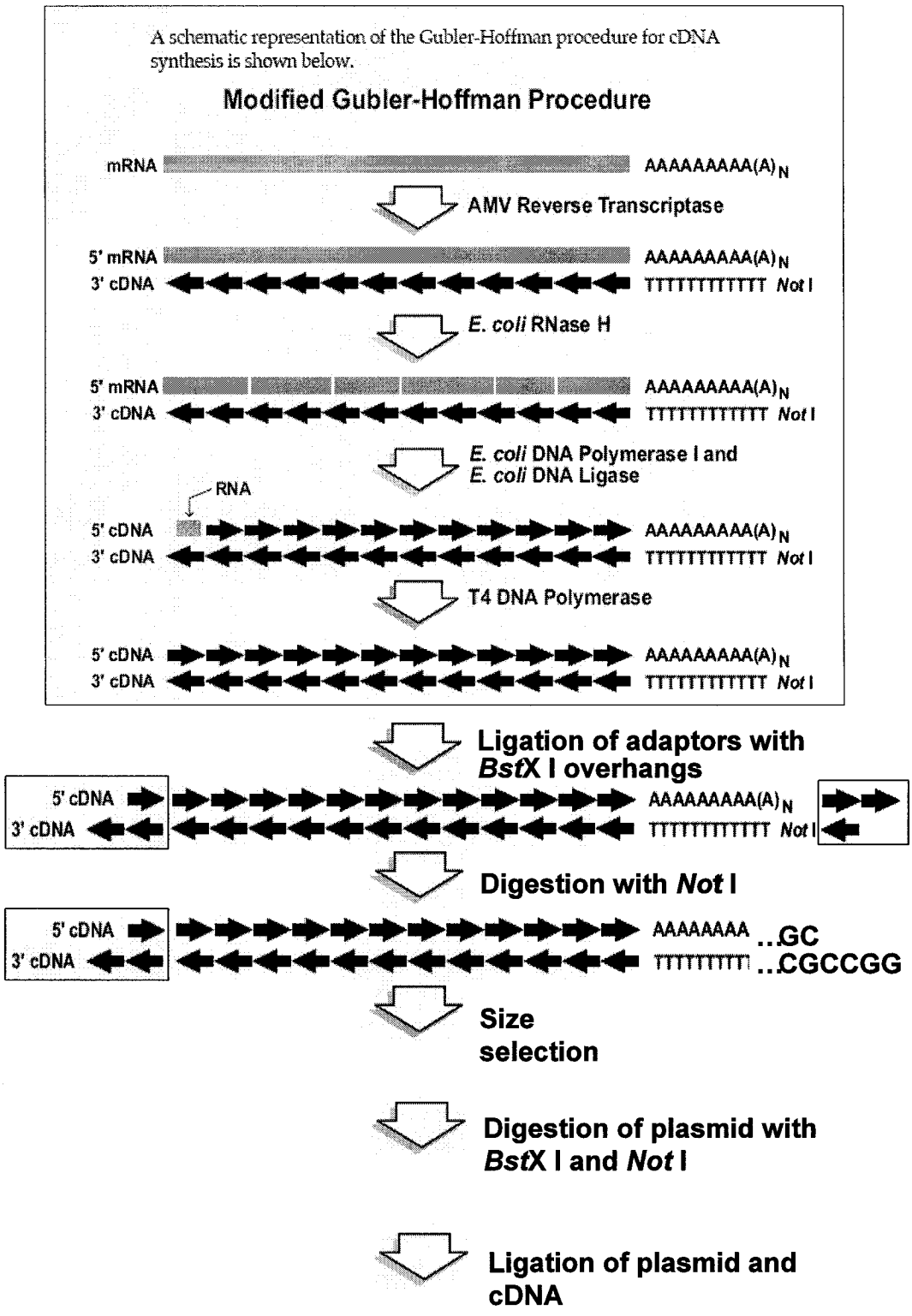


Figure 3.2 HEK293 cells show the lowest binding of ^{125}I-ASP: Cells were seeded into 96 well tissue culture plates the day before the binding assay. Cells were incubated in serum free medium for two hours prior to the experiment. ^{125}I-ASP (1 nM) plus increasing concentrations of competing cold ASP were added in 100 µl 2% BSA in PBS. After incubating 1 hour at room temperature, cells were washed three times with 2% BSA. Cell protein was extracted in 0.1 N NaOH, and aliquots taken for counting and assay of total cell protein by the Bradford method. Results shown are representative experiments.

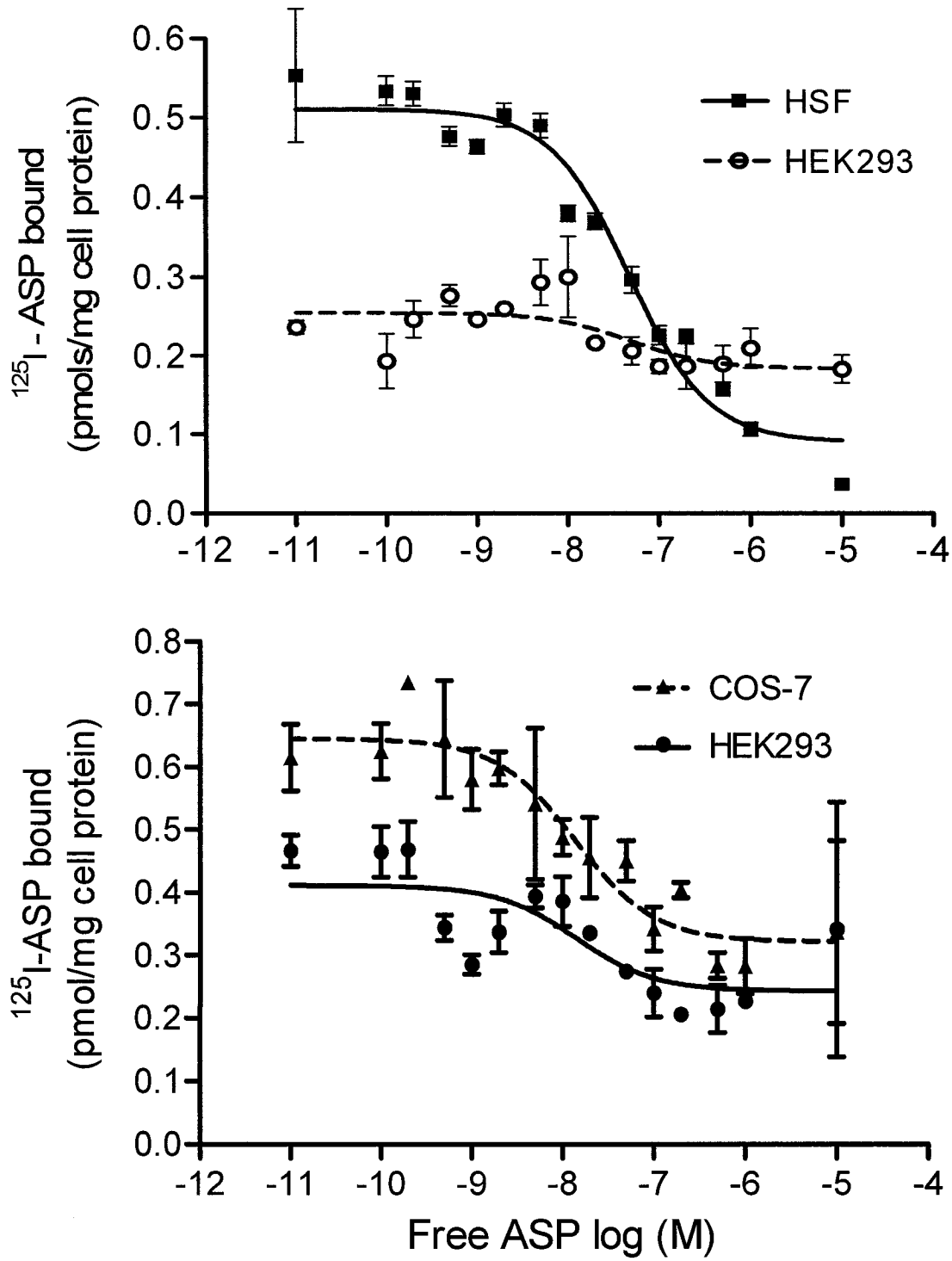
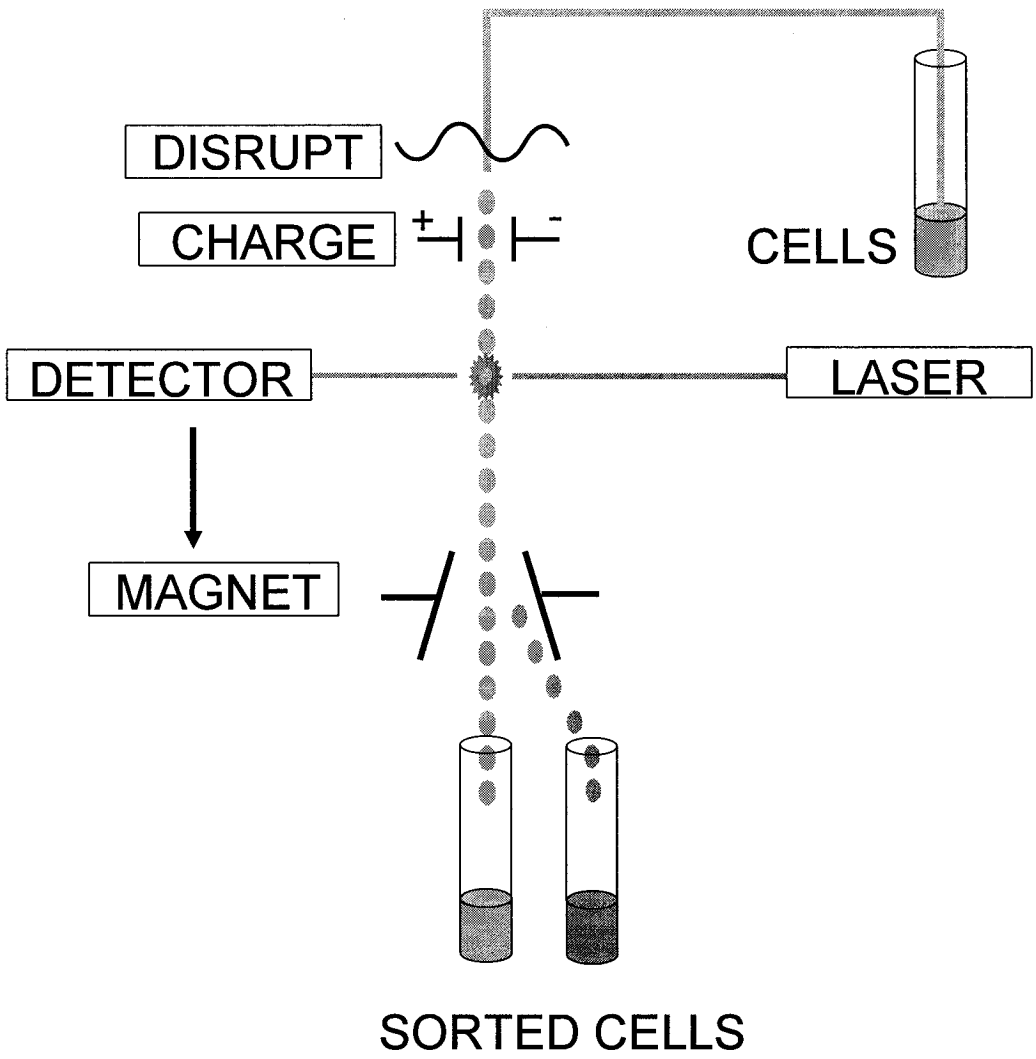

^{125}I - ASP bound
(pmols/mg cell protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-12
-11
-10
-9
-8
-7
-6
-5
-4
HSF
HEK293
^{125}I-ASP bound
(pmol/mg cell protein)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
COS-7
HEK293
Free ASP log (M)

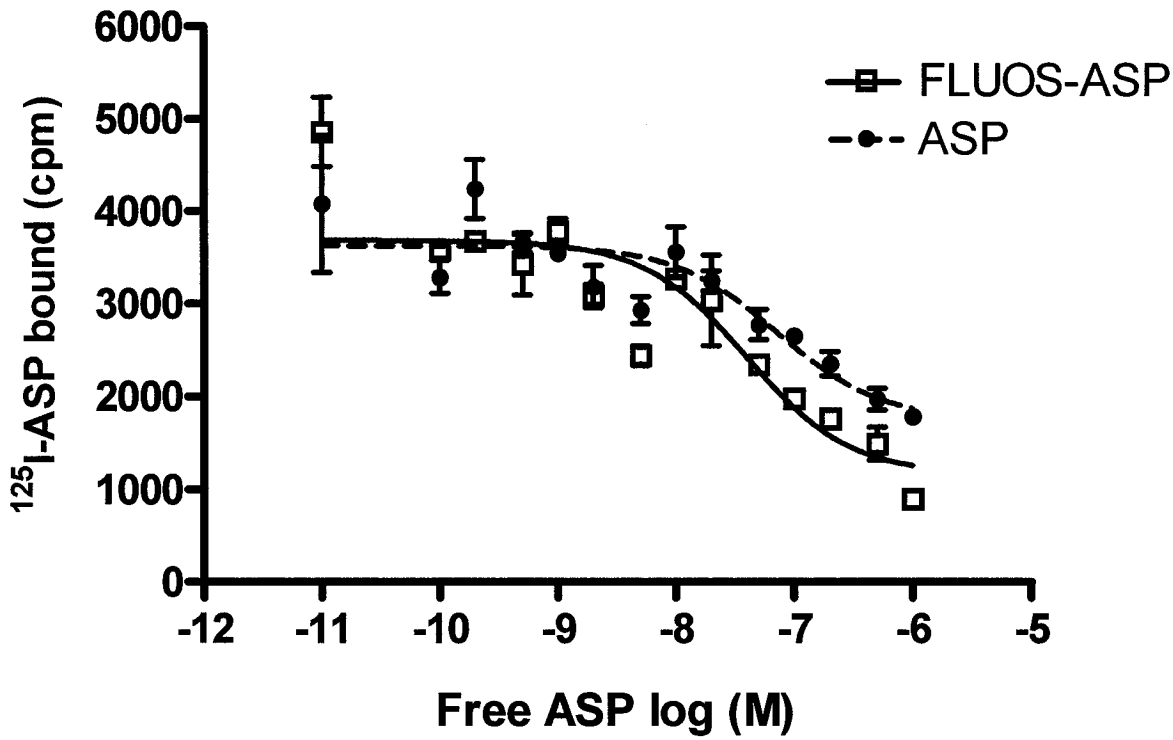
Figure 3.3 Fluorescence activated cell scanning or sorting



In a cell scanner, suspended, fluorescently labeled cells are taken up into a stream of fluid fine enough that cells pass the laser / detector one at a time.

In a sorter, the fluid stream is broken into droplets that each contain a single cell. A charge is added to each droplet before it passes the laser. The detector sends a signal to magnetic plates that are used to divert the droplets, at a level of fluorescence set by the operator.

Figure 3.4 FLUOS labeled ASP binds as effectively as native ASP



Human skin fibroblasts were seeded into 96 well tissue culture plates the day before the binding assay. Cells were incubated in serum free medium for two hours prior to the experiment. ^{125}I-ASP (1 nM) plus increasing concentrations of competing native or FLUOS labeled ASP in 2% BSA in PBS, were added. After incubating 1 hour at room temperature, cells were washed three times with 2% BSA. Cell protein was extracted in 0.1 N NaOH, and aliquots taken for counting and assay of total cell protein by the Bradford method. Results shown are representative experiments.

Figure 3.5 The fluorescence of library transfected cells is not higher than mock transfected cells: HEK293 cells in T75 tissue culture flasks, at 80 to 90% confluency, were transfected with Lipofectamine and 8 μg DNA per flask. Three days later the cells were detached with citrate-saline, centrifuged for 5 minutes at 600g, and resuspended in 2% BSA with 50 nM FLUOS-ASP. After incubating for 1 hour at 37 °C with gentle shaking, cells were placed on ice and sorted by FACS. The histograms shown here are from the second sort.

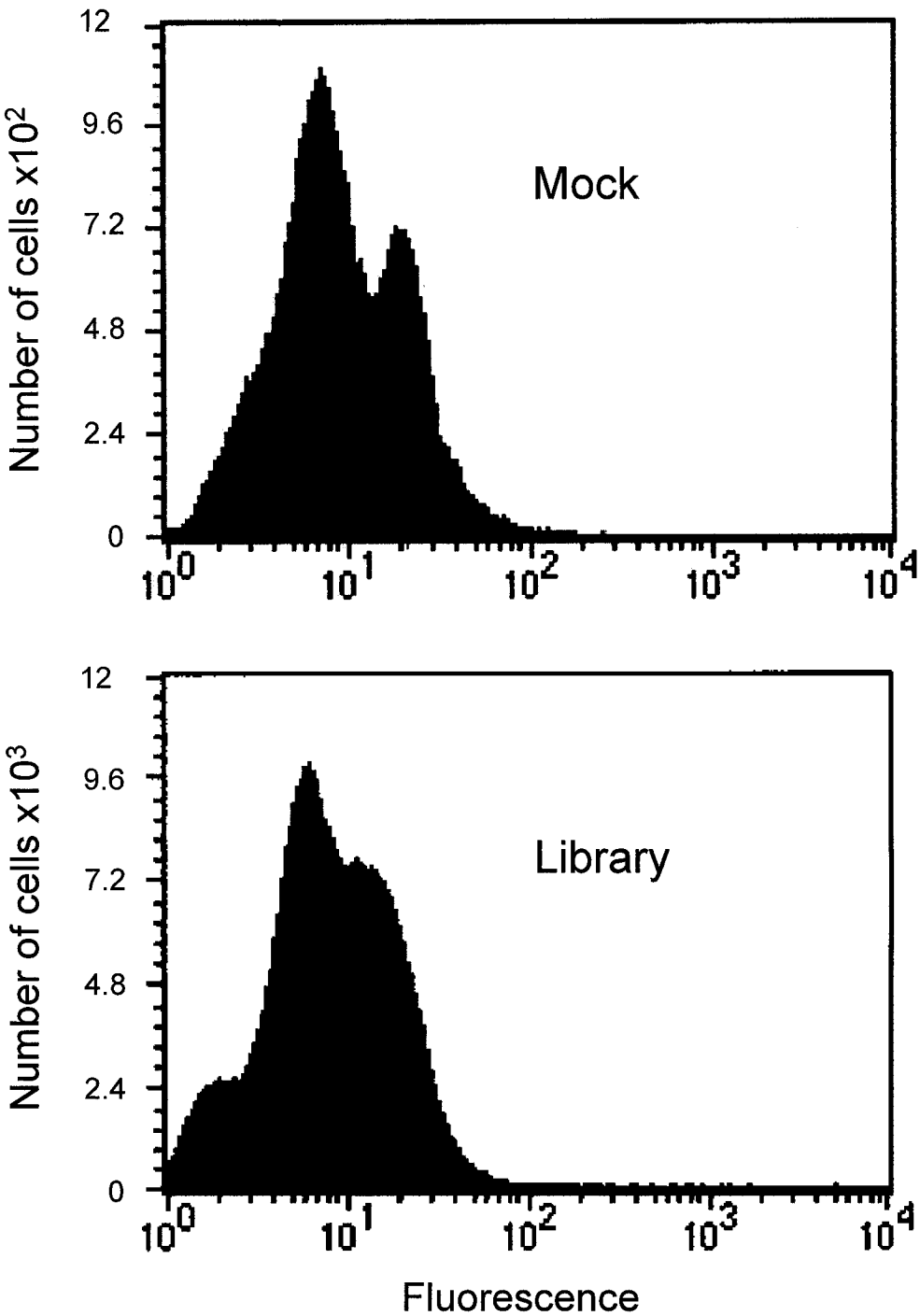

12
9.6
7.2
4.8
2.4
0
Number of cells x10²
Mock
10⁰
10¹
10²
10³
10⁴
12
9.6
7.2
4.8
2.4
0
Number of cells x10³
Library
10⁰
10¹
10²
10³
10⁴
Fluorescence

Figure 3.6 Of The Pools That Bound More ^{125}I-ASP, Pool # 18 Was The Most Consistent: Amplified library DNA, in 40 pools, was transfected into HEK293 cells seeded the day before in 24 well plates. Two days later, the cells were incubated in serum free medium for 2 hours, after which they were incubated with 20 nM ^{125}I-ASP in HAG-CM buffer for one hour at 37 °C. Cells were rinsed three times with HAG-CM, extracted with 0.1 N NaOH, and counted.

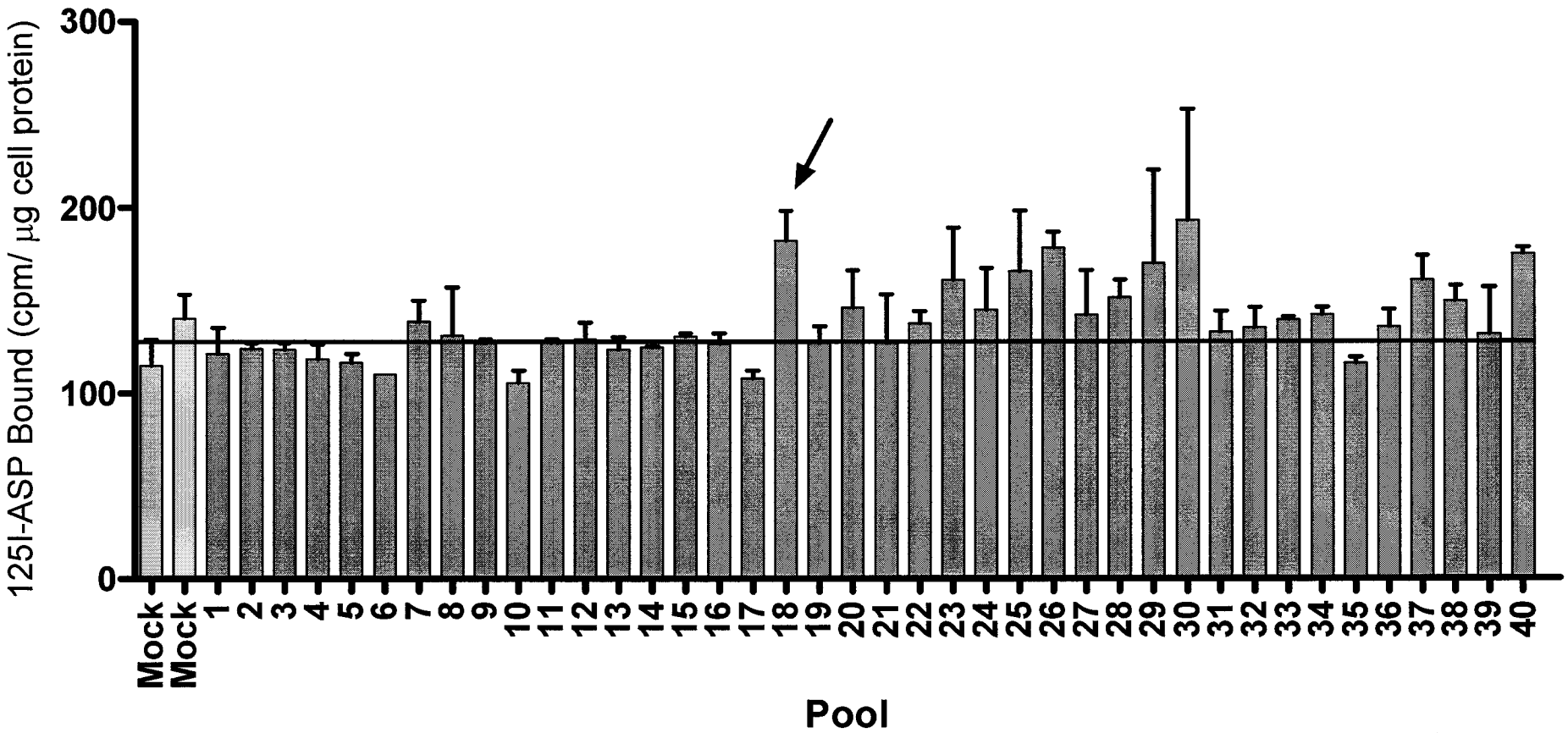

125I-ASP Bound (cpm/ μg cell protein)
300
200
100
0
Mock
Mock
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
Pool

Figure 3.7 Design of degenerate primers

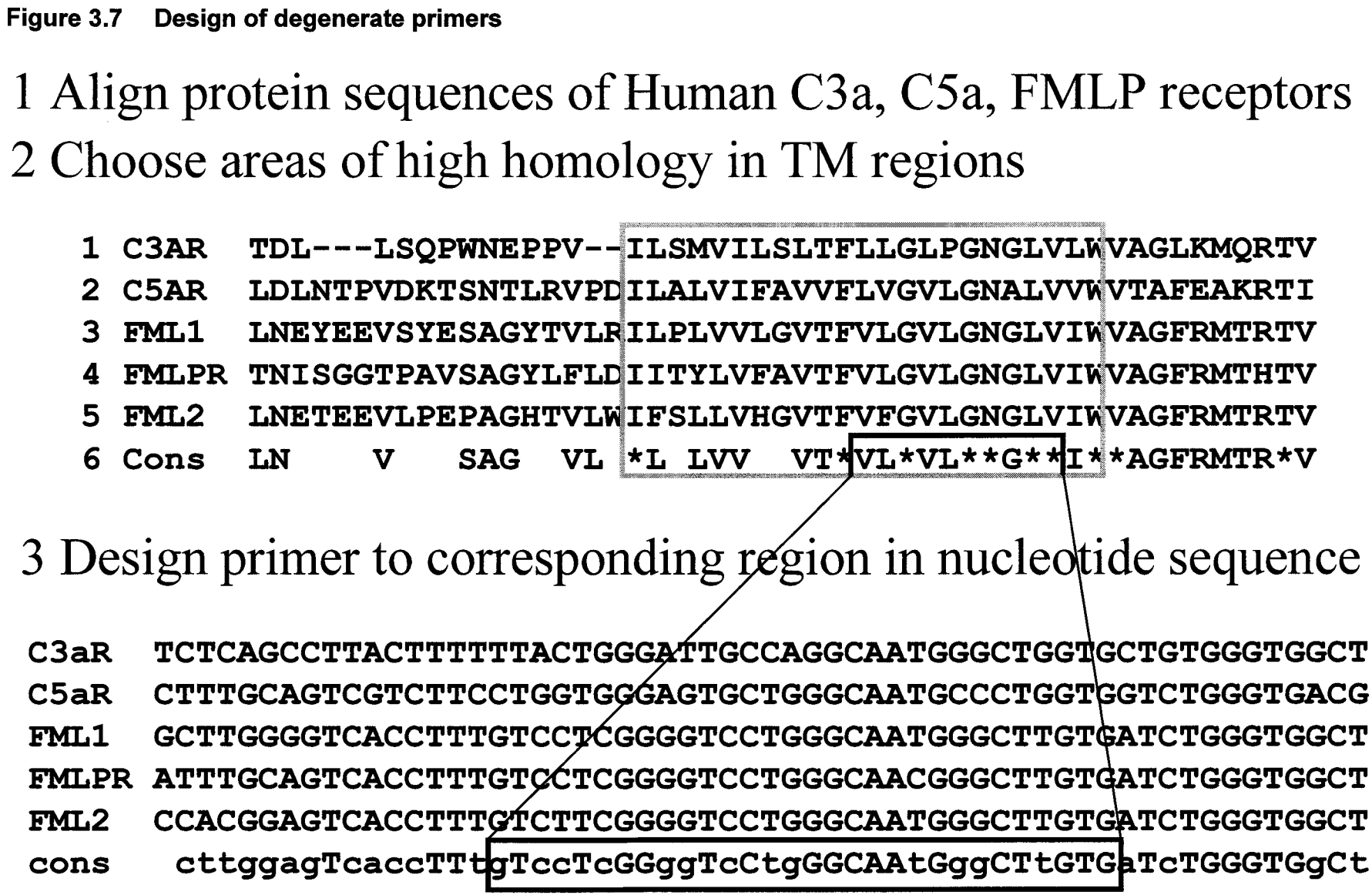
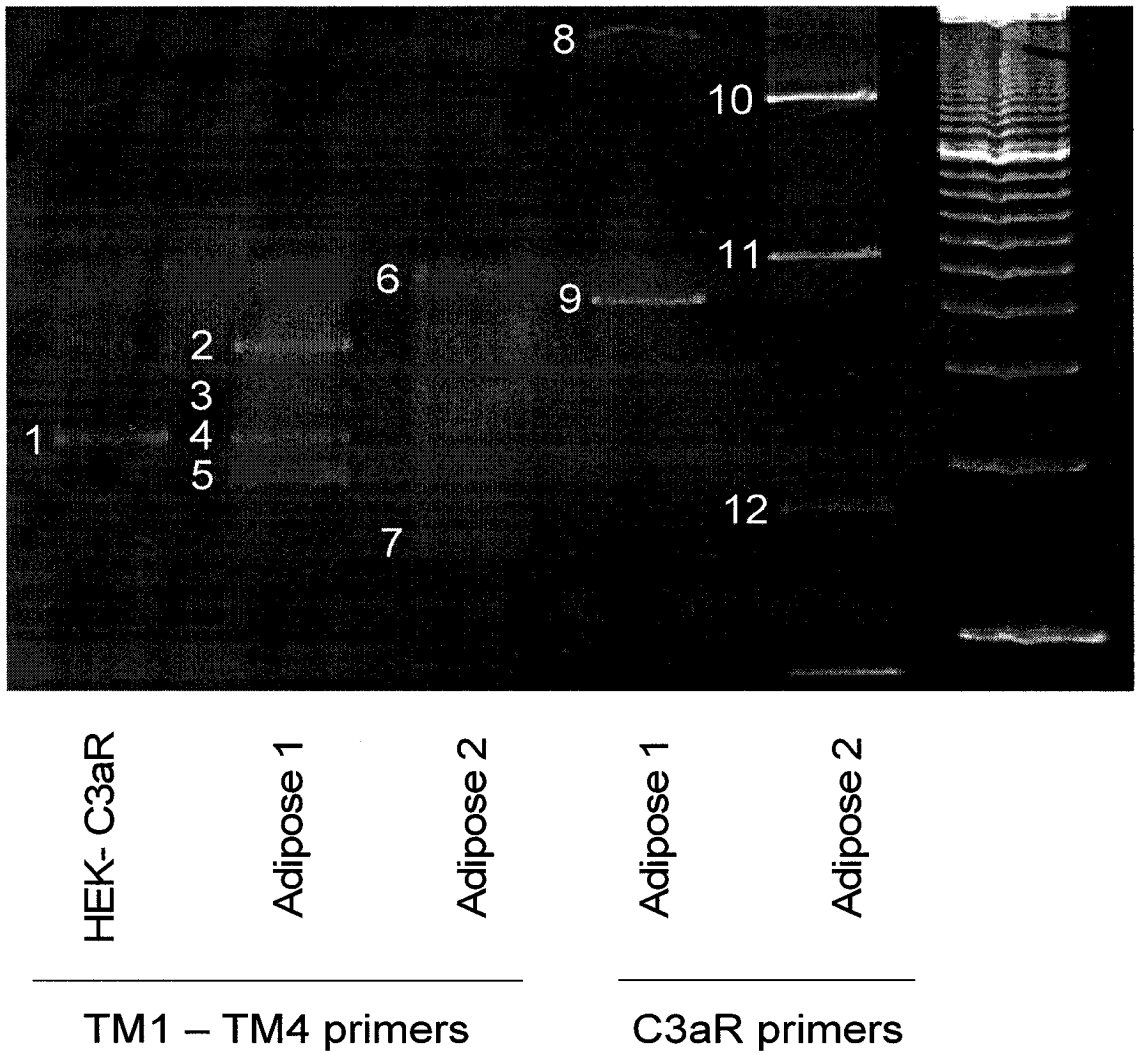


Figure 3.8 The degenerate primers amplified several products from ASP responsive sources



RNA from the adipose tissue of two independent subjects, (sources responsive to ASP), as well as from HEK-C3aR (source not reponsive to ASP) (Table 4.4), were subjected to RT-PCR using the primes for TM1 and TM4, as wells as for C3aR. PCR products were electrophoresed on an agarose gel and visualized with ethidium bromide.

Figure 3.9 Protocol for Isolation of Clones With Probes Designed from Degenerate Primers

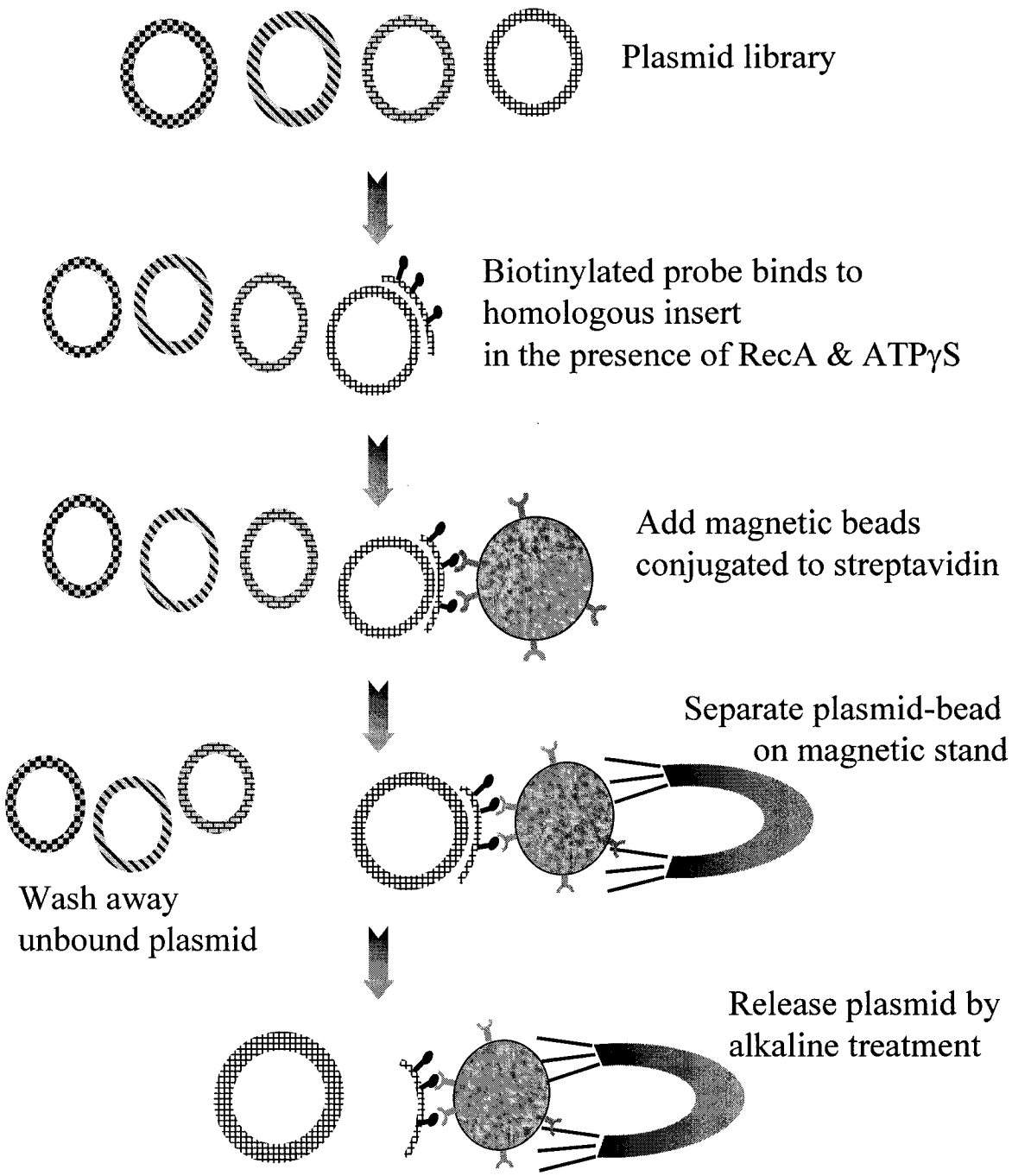
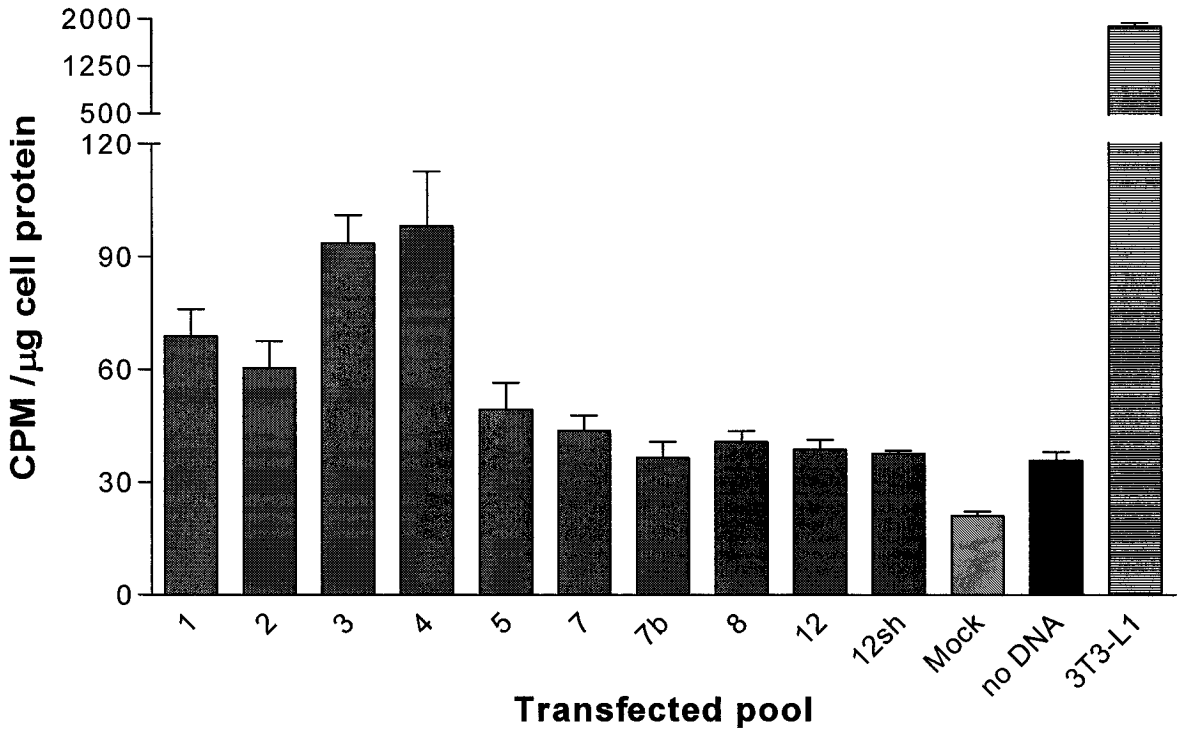
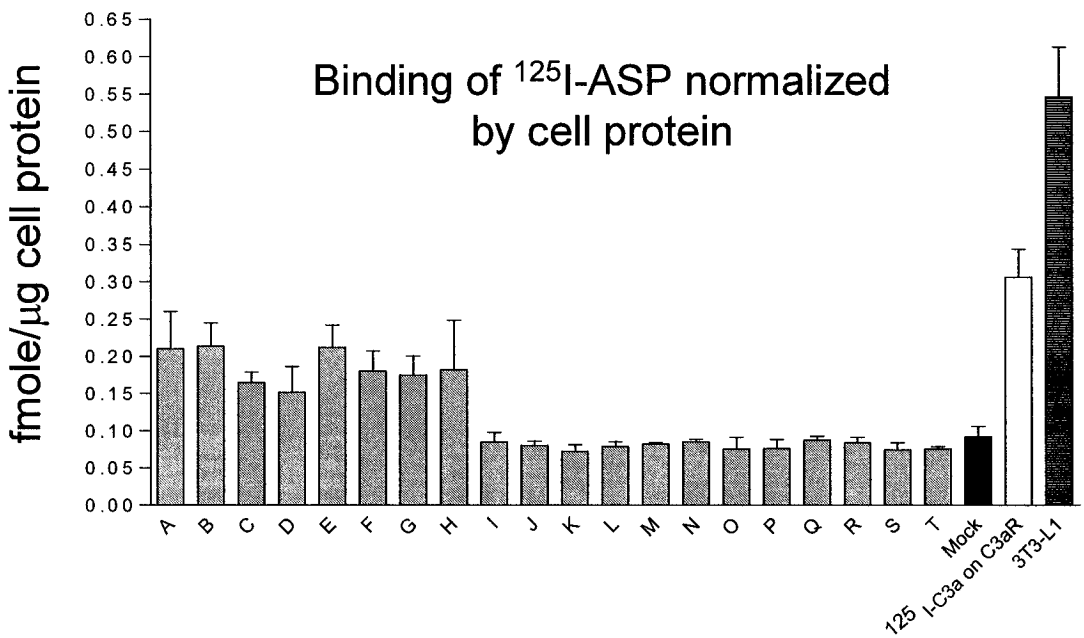


Figure 3.10 Plasmid pools isolated with degenerate probes show increased ASP binding



Pools of plasmids isolated with the magnetic bead protocol, were transfected into HEK293 cells, and assayed 2 days later. Cells were incubated with 1 nM ^{125}I-ASP in HAG-CM buffer for one hour at 37 °C, rinsed three times in HAG-CM, extracted with 0.1 N NaOH and counted. As a positive control, 3T3-L1 cells were incubated with ^{125}I-ASP and treated as for the transfected cells. Mock transfected (empty plasmid) cells were used as a negative control.

Figure 3.11 The subpools that bound more ASP also had lower cell protein: The high binding pools of plasmids isolated with the magnetic bead protocol, were further divided into 20 subpools, transfected into HEK293 cells, and assayed 2 days later. Cells were incubated with 1 nM ^{125}I-ASP in HAG-CM buffer for one hour at 37 °C, rinsed three times in HAG-CM, extracted with 0.1 N NaOH and counted. As a positive control for transfection, C3aR was transfected into HEK293 cells and assayed via binding of ^{125}I-C3a. As a positive control for ASP binding, 3T3-L1 cells were incubated with ^{125}I-ASP and treated as for the transfected cells. Mock transfected (empty plasmid) cells were used as a negative control.


Binding of 125I-ASP normalized by cell protein
fmole/µg cell protein
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A B C D E F G H I J K L M N O P Q R S T Mock 125I-C3a on C3aR 3T3-L1

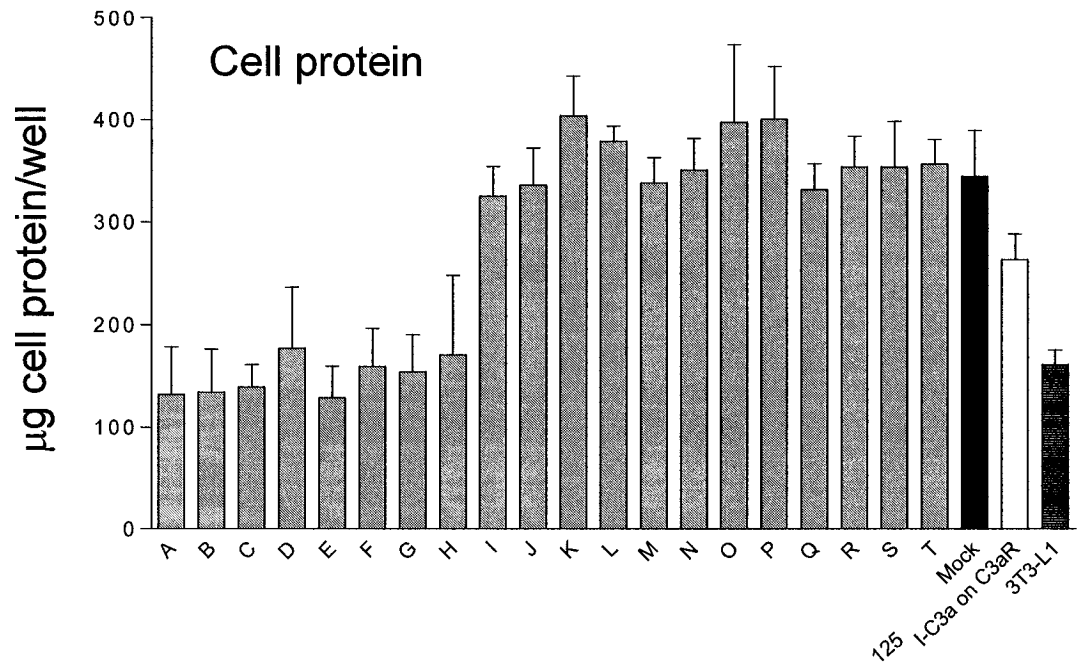
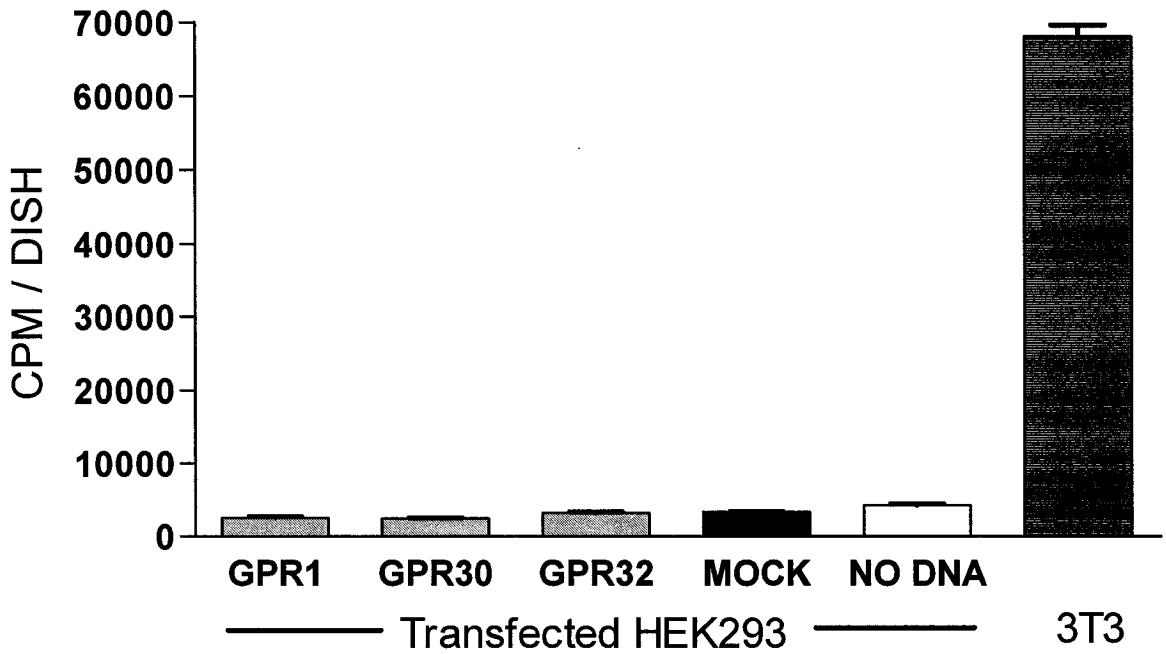

Cell protein
µg cell protein/well
500
400
300
200
100
0
A B C D E F G H I J K L M N O P Q R S T Mock 125I-C3a on C3aR 3T3-L1

Figure 3.12 Binding of 125I-ASP is not increased in HEK293 cells transfected with orphan receptors: In two separate experiments, plasmids containing orphan receptor cDNAs were transfected into HEK293 cells, and assayed for binding/uptake of 1 nM ^{125}I-ASP two days later. Binding/uptake of ^{125}I-ASP to 3T3-L1 cells (both panels), and of ^{125}I-C3a to C3aR-expressing HEK293 cells (bottom panel), are shown as controls. Mock transfected (empty plasmid) cells were used as a negative control.


70000
60000
50000
40000
30000
20000
10000
0
CPM / DISH
GPR1
GPR30
GPR32
MOCK
NO DNA
Transfected HEK293
3T3

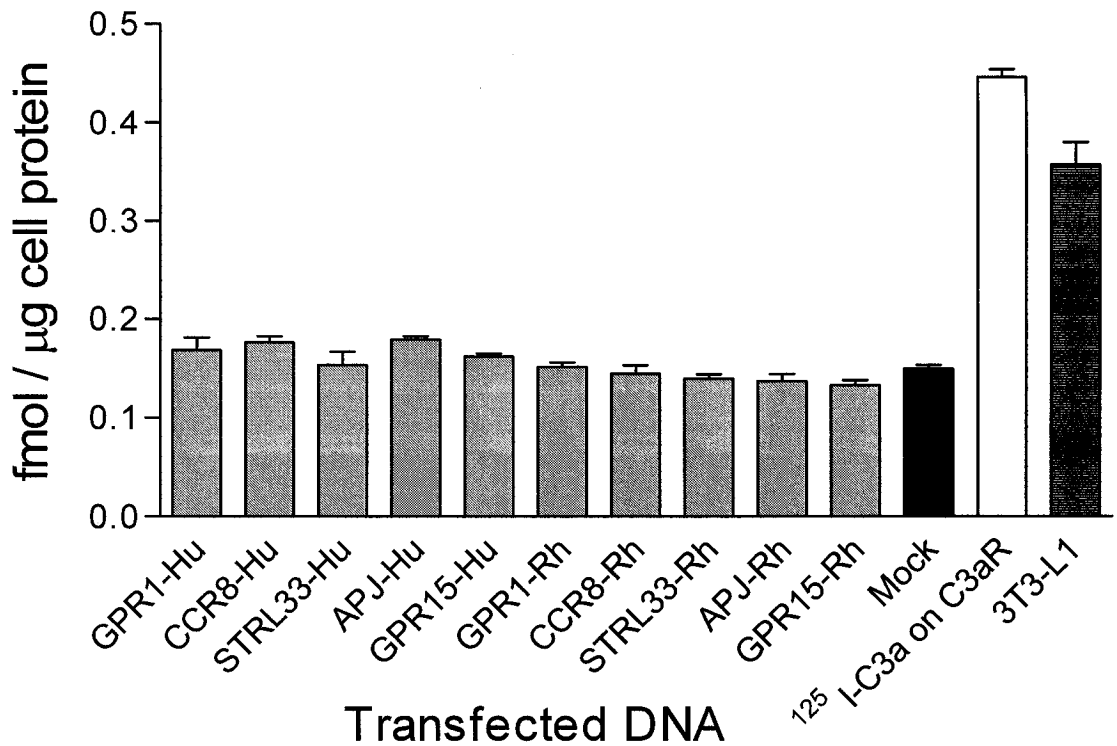
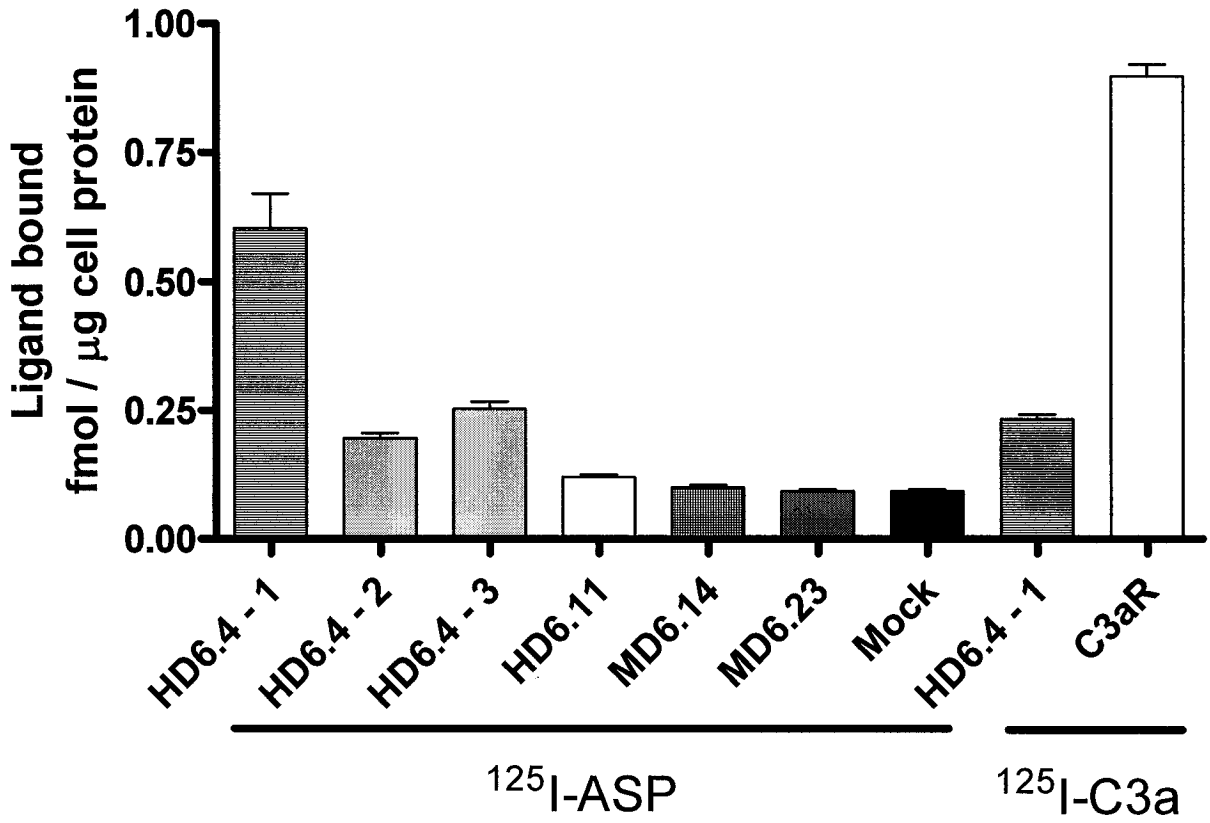
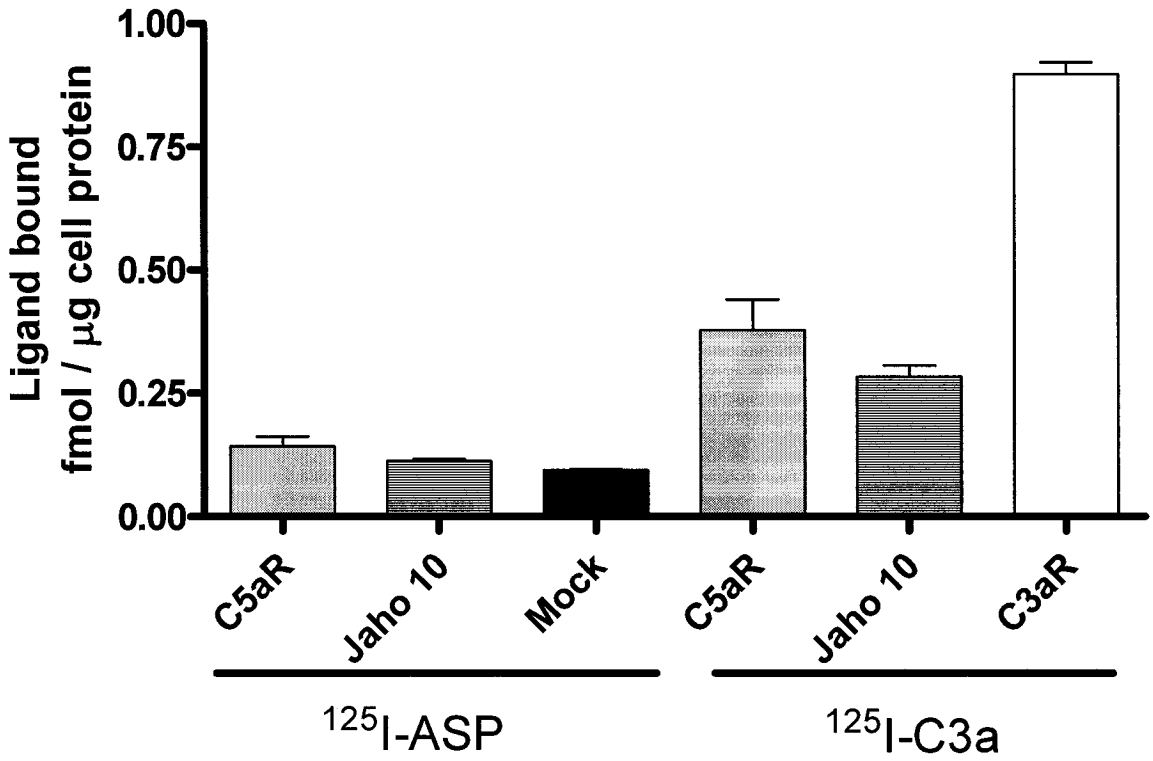

0.5
0.4
0.3
0.2
0.1
0.0
fmol / µg cell protein
GPR1-Hu
CCR8-Hu
STRL33-Hu
APJ-Hu
GPR15-Hu
GPR1-Rh
CCR8-Rh
STRL33-Rh
APJ-Rh
GPR15-Rh
Mock
125 I-C3a on C3aR
3T3-L1
Transfected DNA

Figure 3.13 Clone HD6.4 shows inconsistent binding of ASP, and no binding of C3a



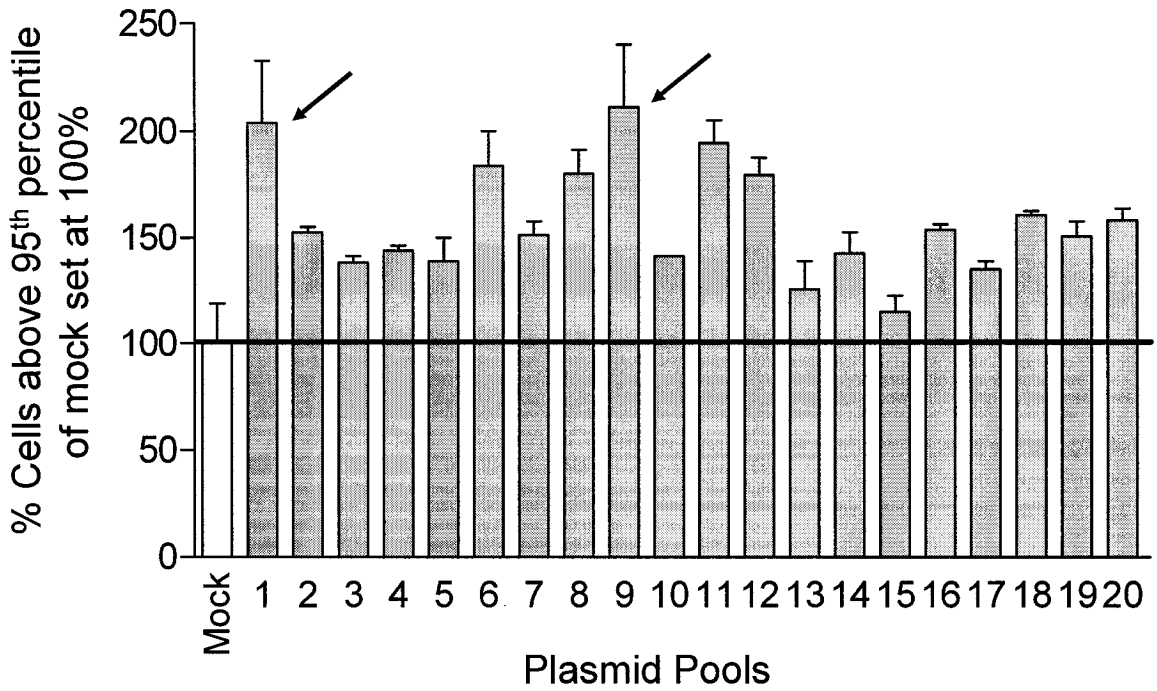
HEK293 cells stably transfected with cDNAs for orphan receptors or C3aR were seeded into 24 well plates. Cells were incubated for 2 hours in serum free medium and assayed for binding/uptake of 1 nM ^{125}I-ASP (orphan receptors) or 1 nM ^{125}I-C3a (HD6.4 and C3aR). Mock transfected (empty plasmid) cells were used as a negative control.

Figure 3.14 Neither ^{125}I-ASP nor ^{125}I-C3a bind to C5aR



HEK293 cells were transfected with plasmids containing C5aR, Jaho10, or C3aR. Two days later cells were assayed for binding/uptake of ^{125}I-ASP or ^{125}I-C3a as indicated. Mock transfected (empty plasmid) cells were used as a negative control.

Figure 3.15 Two 1st round pools show increased FLUOS-ASP binding in transfected HEK293 cells



After 2 rounds of sorting library-transfected cells, recovered plasmid was amplified in 20 separate pools. HEK293 cells in 6 well plates were transfected with 4 μg DNA per well and assayed 2 days later by binding/uptake of FLUOS-ASP. Binding/uptake above the 95th percentile in mock transfected (empty plasmid) cells was considered background, and set at 100%. Arrows indicate the 2 pools that were further subdivided.

Figure 3.16 Subpools show enrichment of ASP binding in transfected HEK293 cells: Two pools were each divided into 20 subpools, and assayed along with the respective parent pools for FLUOS-ASP binding/uptake. Binding/uptake above the 95th percentile in mock transfected (empty plasmid) cells was considered background, and set at 100%. Arrows indicate the subpools showing the highest binding/uptake.

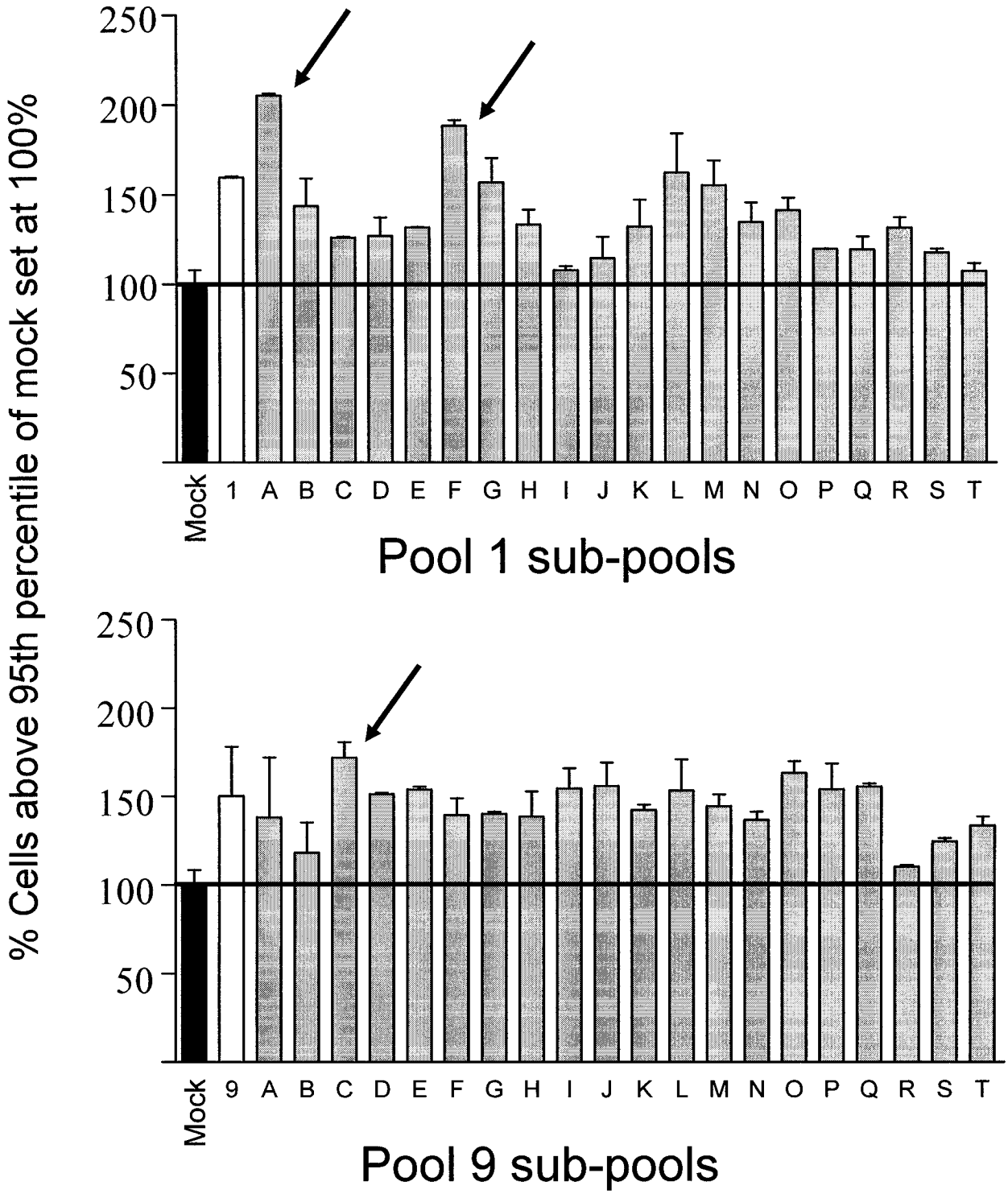
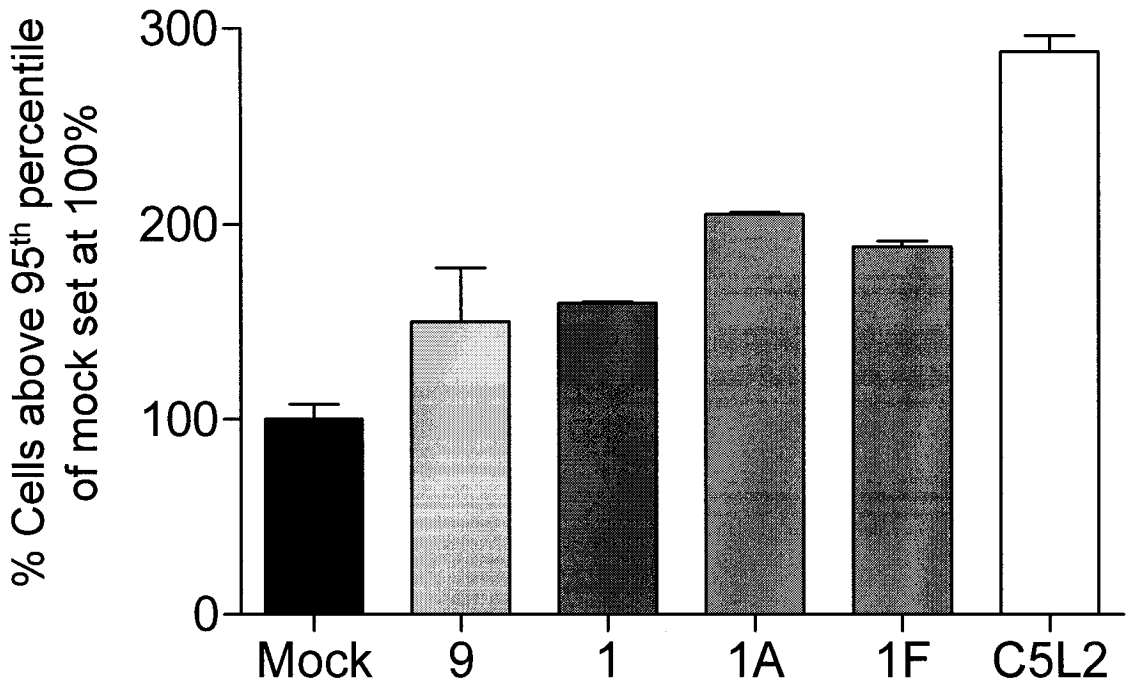

% Cells above 95th percentile of mock set at 100%
250
200
150
100
50
Mock
1 A B C D E F G H I J K L M N O P Q R S T
Pool 1 sub-pools
250
200
150
100
50
Mock
9 A B C D E F G H I J K L M N O P Q R S T
Pool 9 sub-pools

Figure 3.17 HEK293 cells transfected with the C5L2 cDNA bind more FLUOS-ASP than positive library pools



Plasmids containing the cDNA for the orphan receptor C5L2, as well as the 2 positive library pools and 2 subpools, were transfected into HEK293 cells. Two days later the binding/uptake of FLUOS-ASP was assessed. Binding/uptake above the 95th percentile in mock transfected (empty plasmid) cells was considered background, and set at 100%.

PREFACE

The previous chapter detailed the various strategies employed to clone the ASP receptor. Although the initial library screening approach, using improved techniques, was working, the first binding tests of the orphan receptor C5L2 indicated that it might indeed be an ASP receptor. Repeated binding experiments on transiently transfected HEK293 cells confirmed the initial results. The following chapter presents further evidence that C5L2 is an ASP receptor.

In this chapter several new terms are introduced. The immune complement proteins, C4 and C5, can associate with C3b (see chapter 2) to form an attack complex that causes lysis of foreign and damaged endogenous cells. C5 can be cleaved to form C5a, which, along with C3a (see chapter 2), are involved in several immunological responses, as indicated below. On the other hand, C4a has no known function or receptor. Carboxypeptidase B can cleave the C-terminal arginine from C3a, C4a and C5a to produce the desArg forms. The functional capacity of these forms is indicated below. One of the actions of both C3a and C5a, is the stimulation of the fusion of intracellular vesicles with the plasma membranes of myeloid cells. This process is termed degranulation. Degranulation releases several enzymes, including β-hexosaminidase, which aid in the destruction of both foreign and damaged endogenous cells.

The responses to C3a and C5a, are mediated by the binding of these proteins to G protein-coupled receptors (GPCRs). This process, as well as the subsequent activation of a heterotrimeric G protein, are discussed further in chapter 6.

CHAPTER FOUR

THE CHEMOATTRACTANT RECEPTOR-LIKE PROTEIN, C5L2, BINDS C3A DESARG^{77}/ACYLATION STIMULATING PROTEIN

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David Kalant[1,2*], Stuart A. Cain[3*], Magdalena Maslowska[1*], Allan D Sniderman[1], Katherine Cianflone[1] and Peter N. Monk[3]

[1]Mike Rosenbloom Laboratory for Cardiovascular Research, Division of Medicine, McGill University Health Centre, Montreal, Quebec, H3A 1A1, Canada;
[2]Department of Chemistry/Biochemistry, Concordia University, Montreal, Quebec, H4B 1R6, Canada
[3]Department of Neurology, University of Sheffield Medical School, Sheffield, S10 2RX, UK.

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

Address correspondence to:

Peter N Monk
Department of Neurology, E Floor
University of Sheffield Medical School
Beech Hill Road, Sheffield
S10 2RX, UK
Tel. +44 114 2261312
Fax: +44 114 2760095
Email: p.monk@shef.ac.uk

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* These authors contributed equally to this work.

Abbreviations:

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.

Summary

Recently, the orphan receptor C5L2 has 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 $G\alpha_{16}$. 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 the 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.

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 (325) and protection against toxic insult (326-329). C5a and C3a are also reported to have psychopharmacological effects on feeding and drinking behavior (125,330). 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 (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 (331) and, unlike C3a, C3a desArg^{77} does not stimulate eosinophil chemotaxis (332), prostanoid production by guinea pig peritoneal macrophages and rat Kupffer cells (333) or human monocyte-like U937 cell degranulation (257). However, responses to C3a desArg^{77} have been reported: cytotoxicity of NK cells is inhibited by both C3a and C3a desArg^{77} (334); cytokine production by human monocyte/macrophages and PBMC is enhanced by these ligands but inhibited in human tonsil-derived B cells (335,336) and histamine release from rat peritoneal mast cells is stimulated (337). In addition, 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 (201). This triglyceride stimulating activity is also shared by C3a (90). 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 characterised a novel chemoattractant binding protein, C5L2, that has high affinity for C5a, C5a desArg^{74} and C3a (338). Here we report that C5L2 also binds C3a desArg^{77}/ASP and is expressed in three C3a desArg^{77}/ASP-responsive cell types.

Experimental Procedures

Cell Lines and Culture Conditions. HSF were obtained as described previously (339). RBL-2H3, HEK293, 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 HEK293 stably transfected cells.

Stable Transfection of RBL and HEK293 Cells. C5L2, C3aR and CD88-expressing RBL-2H3 and HEK293 cells were produced as described (338). $G\alpha_{16}$ was cloned from human monocyte mRNA and authenticated by sequencing. Human $G\alpha_{16}$ 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 (340). 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. HEK293 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.

Transient Transfection of HEK293 Cells. HEK293 cells were seeded into 6-well plates at 1 x 10^6 cells/well the day before transfection. The C5L2 cDNA in vector pEE6hCMV.neo (Celltech) or the C3aR cDNA 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.

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 (341). 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 (90).

Fluorescent Labelling of C3a desArg^{77}/ASP *and C3a.* C3a desArg^{77}/ASP and C3a were labelled 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.

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-expressing RBL cells in 96-well microtiter plates (55,000 cells/well) at 4°C as described previously (342). Competition assays for HSF, 3T3-L1, U937 and HEK293 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.

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 (325). The serum recognised RBL cells transfected with the cDNA for 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.

Fluorescence - Activated Cell Scanning For Ligand Binding/Uptake Assays. Cells were incubated with the indicated concentrations of FLUOS-labelled C3a desArg^{77}/ASP or C3a for 30 minutes at 37 °C in binding buffer (255) 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 °C for 60 minutes. Again, cells were pelleted, washed twice with PBS and resuspended in FITC-labelled anti-rabbit IgG, (Sigma Chemicals, St. Louis) at (1/1000 dilution in 3% BSA in PBS) and incubated at 4 °C for 60 minutes. Finally, cells were pelleted, washed twice and resuspended in 0.3% paraformaldehyde in PBS for FACS analysis.

Cellular Activation Assays. Cellular activation was measured as the release of β-hexosaminidase from RBL intracellular granules (343) or as stimulation of triglyceride synthesis in HSF and 3T3-1 cells (90). 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.

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 the human C5L2 cDNA were: sense 5'-CCTGGTGGTCTACGGTTCAG-3' and antisense 5'-GGGCAGGATTTGTGTCTGTT-3' (product size 798 bp). Primers for the murine C5L2 cDNA (Ensembl gene ID: ENSMUSG00000041388) were: sense 5'-ATGGCCGACTTGCTTTGT-3' and antisense 5'-CCTTGGTCACCGCACTTTC-3' (product size 739 bp). 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.

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 (338). Here we show that the desArg[77] forms of C4a and C3a also are ligands for this receptor when expressed in the RBL-2H3 cell line (Figure 4.1A,

4.1B, Table 4.1) and can compete strongly with ^{125}I-C3a for C5L2 binding (Figure 4.1A). In contrast, C4a desArg^{77} and C3a des-Arg^{77}/ASP cannot compete effectively with ^{125}I-C5a for C5L2 or CD88 binding (Figure 4.1B, Table 4.1). Although C3aR and C5L2 bind C3a with similar affinities, C3aR has no detectable affinity for C3a desArg^{77}/ASP (Table 4.1). 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 4.1). The data suggest either that C5L2 has two conformations with different ligand binding profiles or that the receptor has two binding sites. As we previously have shown that the Bmax values for ^{125}I-C3a and ^{125}I-C5a binding to C5L2-expressing RBL cells are identical (338), 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 labelled with FLUOS. Increasing

concentrations of C3a desArg^{77}/ASP were incubated with HEK293 cells transiently transfected with the cDNA for C5L2, and binding and uptake were assessed by flow cytometry (Figure 4.2A). FLUOS-C3a desArg^{77}/ASP clearly binds to C5L2, with half maximal fluorescence intensity at approximately 3 nM, whereas mock-transfected cells (Figure 4.2A 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 HEK293 cells transiently transfected with the C3aR cDNA is shown (Figure 4.2B), 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 (Figure 4.2C).

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 HEK293 cells transfected with C3aR and CD88. ^{125}I-C3a desArg^{77}/ASP does not bind to C3aR-transfected HEK293 cells and does not compete with ^{125}I-C3a (Table 4.2), as found previously (344). 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 HEK293 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 (345), bind both ^{125}I-C3a desArg^{77}/ASP and ^{125}I-C3a with high affinity (Table 4.2). As observed in C5L2-expressing 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-expressing RBL cells (Tables 4.2 and 4.1, respectively), while C3a was an effective competitor for ^{125}I-C3a desArg^{77}/ASP binding (Table 4.2). Thus, C5L2 has not only 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. Previously, we have shown that C5a, C5a desArg^{74}, C4a or 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 (338). 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 10μM (data not shown). As well, there was no effect of these two ligands on either CD88 or C3aR activation of degranulation (Table 4.3) 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-expressing 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} (346) and also to the toxin-insensitive G_q-family member, G_{16} (347,348). 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 cDNAs for 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 the CD88 cDNA alone (Fig 4.3A), increasing concentrations of PT inhibit the degranulation response. In co-transfected cells (Fig 4.3B), 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 $\beta\gamma$ subunits and ADP-ribosylated $G\alpha_i$. In C5L2+$G\alpha_{16}$ co-expressing cells, treatment with high concentrations (1 μM) of intact or desArg complement fragments still does not stimulate degranulation (Fig 4.3C). 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 4.4). C3a desArg^{77}/ASP appears to act via stimulation of the protein kinase C pathway (262), and stimulation of this pathway by the phorbol ester PMA also results in increased TGS (Table 4.4). 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 4.4). Increased TGS is not simply a response to C3a binding, however, as C3aR-transfected HEK293 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 4.4). However, these cell types may lack all or part of the signaling pathway necessary to mount an increase in TGS, as there is no significant response to treatment with PMA or insulin (Table 4.4).

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 already has 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 4.5) 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 (343,349). The two binding sites tentatively identified on C5L2 also may 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

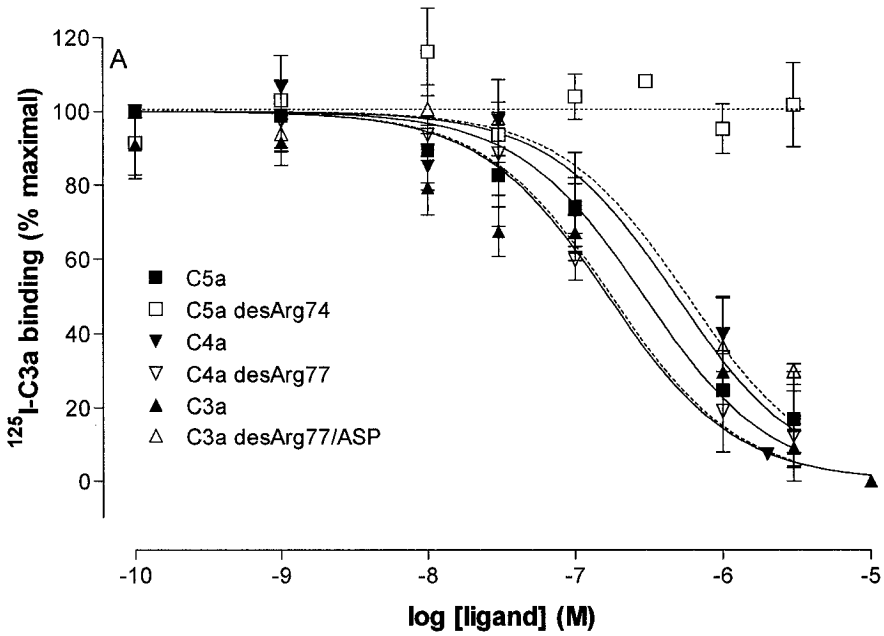
binding to the second site is presumably dependent on the C-terminal Arg residue, as C5a desArg^{74} cannot compete for ^{125}I-C3a binding to C5L2.

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 (196). 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 4.4) and glucose transport (90). 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 gene transcript (Figure 4.4). 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 for C5L2. By contrast, RT-PCR of RNA from the human monocytic cell line U937 and non-transfected HEK293 cells did not result in any PCR product using C5L2 primers in spite of equal levels of glyceraldehyde-3-phosphate dehydrogenase amplification product in parallel reactions (Figure 4.4).

These results were further confirmed using an antiserum specific to the N terminal region of human C5L2. FACS analysis clearly demonstrates that HSF (Figure 4.5A) express endogenous C5L2 on their cell surface although the fluorescent intensity was lower than that of HEK293 cells overexpressing the stably transfected C5L2 cDNA (Figure 4.5B). In contrast, untransfected HEK293 cells did not bind the anti-serum (Figure 4.5C). Since the antiserum does not appear to recognize murine C5L2, cells transfected with the mouse C5L2 cDNA 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 may 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.

Figure 4.1 C3a desArg^{77}/ASP and C4a desArg^{77} bind to RBL cells expressing C5L2: RBL cells stably transfected with the C5L2 cDNA 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
^{125}I-C3a binding (% maximal)
120
100
80
60
40
20
0
C5a
C5a desArg74
C4a
C4a desArg77
C3a
C3a desArg77/ASP
-10
-9
-8
-7
-6
-5
log [ligand] (M)

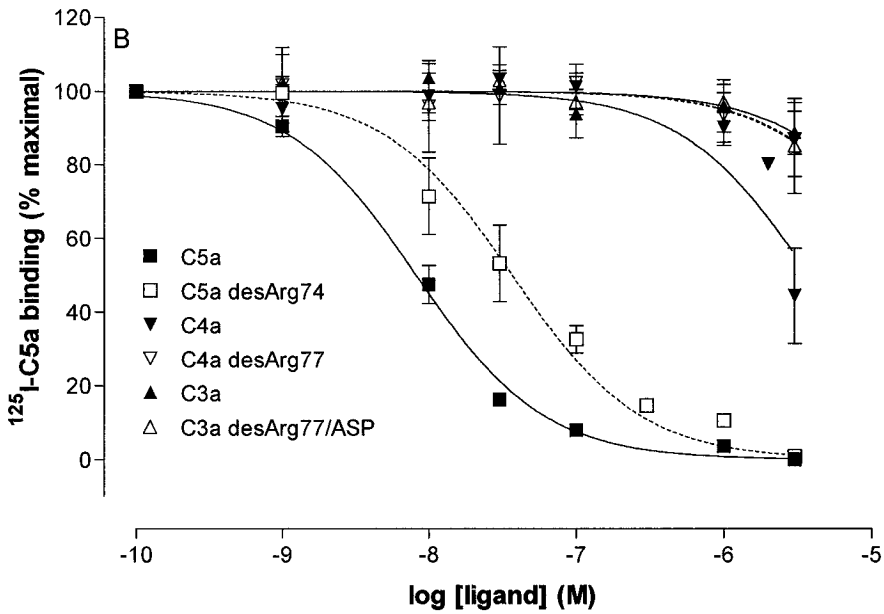

B
^{125}I-C5a binding (% maximal)
120
100
80
60
40
20
0
C5a
C5a desArg74
C4a
C4a desArg77
C3a
C3a desArg77/ASP
-10
-9
-8
-7
-6
-5
log [ligand] (M)

Table 4.1 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.

Figure 4.2 C5L2 shows saturable binding/uptake of C3a desArg^{77}/ASP: HEK293 cells were transiently transfected with the cDNAs for C5L2 (A), C3aR (B) or CD88 (C) and three days later cells were incubated for 30 min with the indicated concentrations of FLUOS-labelled 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).

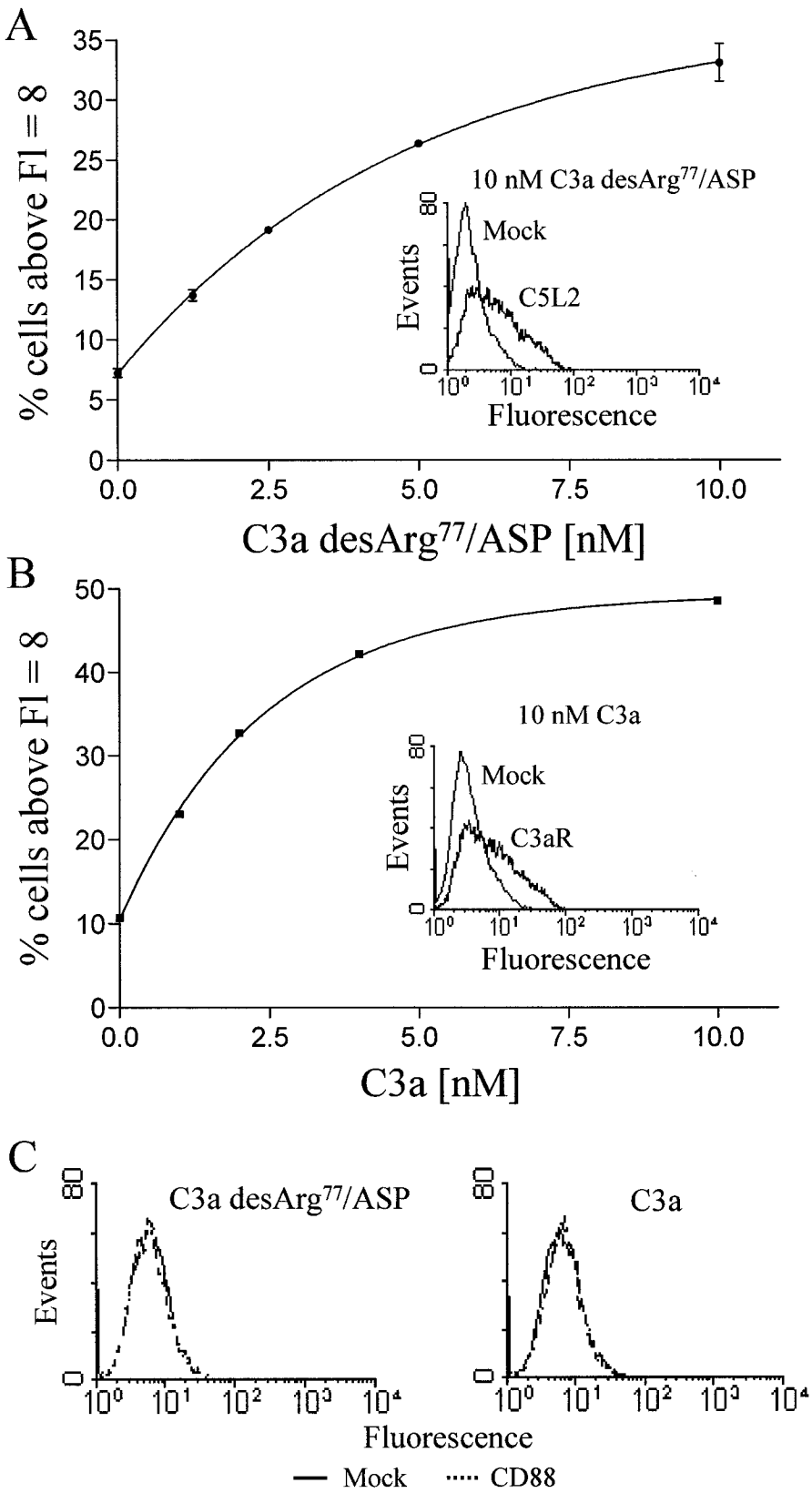

A
% cells above F1 = 8
C3a desArg77/ASP [nM]
10 nM C3a desArg77/ASP
Mock
C5L2
Events
Fluorescence
B
% cells above F1 = 8
C3a [nM]
10 nM C3a
Mock
C3aR
Events
Fluorescence
C
C3a desArg77/ASP
C3a
Events
Fluorescence
Mock
CD88

Table 4.2 Competition Binding Data for Human Skin Fibroblasts and C3aR-expressing HEK293 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_{50}	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 4.3 Summary of Receptor Activation Data for Human Chemoattractant Receptors Expressed in RBL Cells

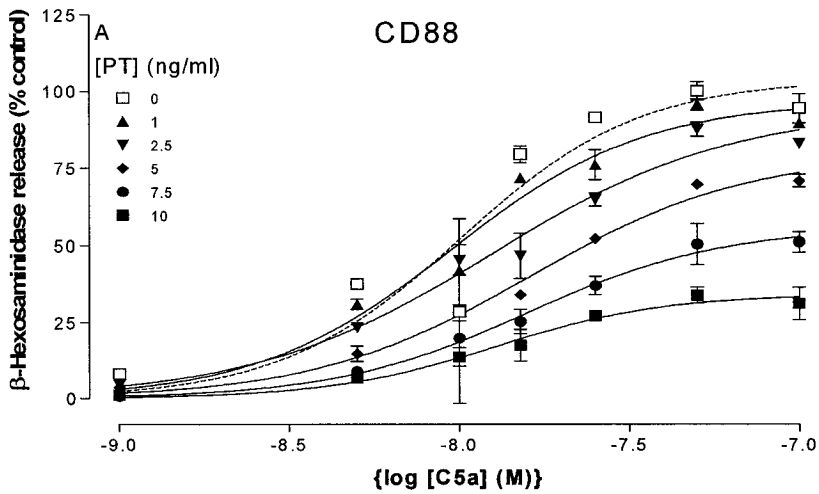
	Receptor			
	CD88		C3aR	
Ligand:	**EC_{50}[1]**	**n[2]**	**EC_{50}**	**n**
C5a	5.85	16	>10,000	2
C5a desArg[74]	21.2	5	>10,000	2
C4a	>10,000	2	>10,000	2
C4a desArg[77]	ND[3]		>10,000	2
C3a	>10,000	2	50.2	8
rC3a desArg[77] /ASP	ND		>10,000	2
Plasma C3a desArg[77]/ASP	>10,000	1	>10,000	2

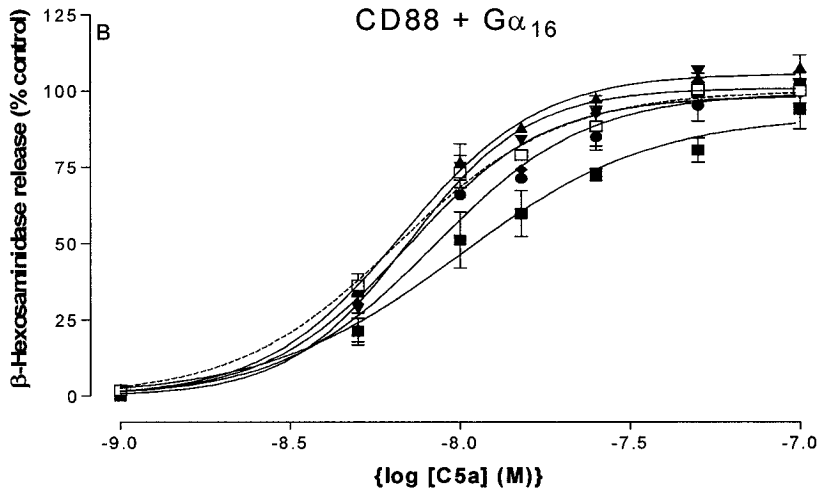
1 EC_{50} = concentration of ligand (nM) resulting in 50% of maximal degranulation.

2 n = number of separate experiments performed in triplicate;

3 ND = assay not done.

Figure 4.3 CD88 but not C5L2 stimulates degranulation coupled to $G\alpha_{16}$ proteins: RBL cells were transfected with cDNAs for 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 β-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 β-hexosaminidase. (C) RBL cells transfected with cDNAs for C5L2+$G\alpha_{16}$ were treated with 1 μM of the indicated complement fragments and degranulation measured as secretion of β-hexosaminidase, expressed as a percentage of the total cellular content.


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)}

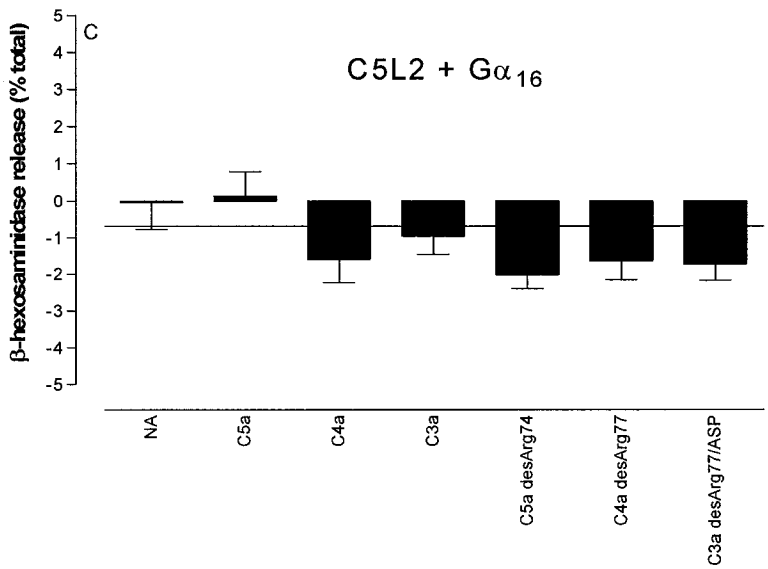

C
C5L2 + Gα16
β-hexosaminidase release (% total)
5
4
3
2
1
0
-1
-2
-3
-4
-5
NA
C5a
C4a
C3a
C5a desArg74
C4a desArg77
C3a desArg77/ASP

Table 4.4 Stimulation of Triglyceride Synthesis in Different Cell Lines.

		Cell Type				
		HSF (n^1 = 5)	U937 (n=3)	Bt2cAMP-U937 (n=3)	HEK293 (n=3)	HEK-C3aR (n=3)
		% of Stimulation of Triglyceride Synthesis[2]				
Stimulus	**Conc**					
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.

4 ND = assay not done. * = significantly different from basal, $p<0.025$.

Table 4.5 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.

Figure 4.4 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 the C5L2 gene 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 (HEK293, 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; HEK293. Lane 4; U937 monocytic cells. Lane 5; 100 bp DNA ladder with 1000 bp indicated. Lane 6; 3T3-L1 preadipocytes.

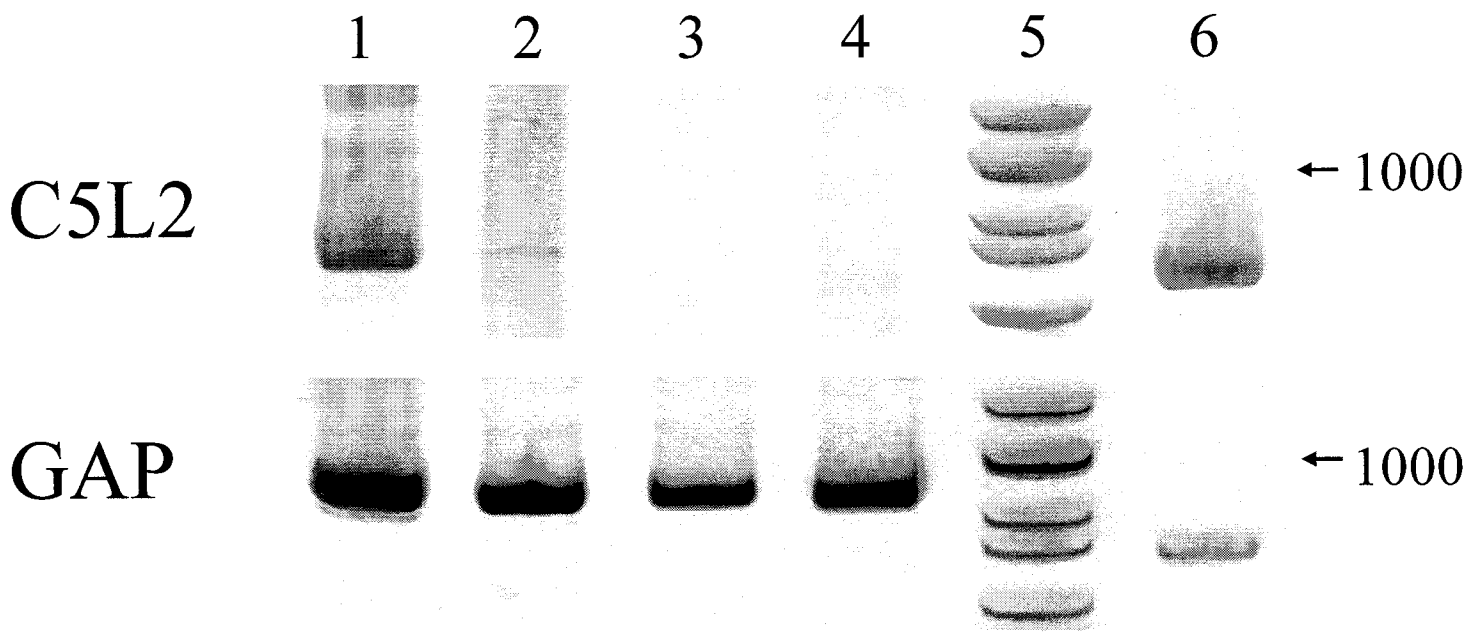
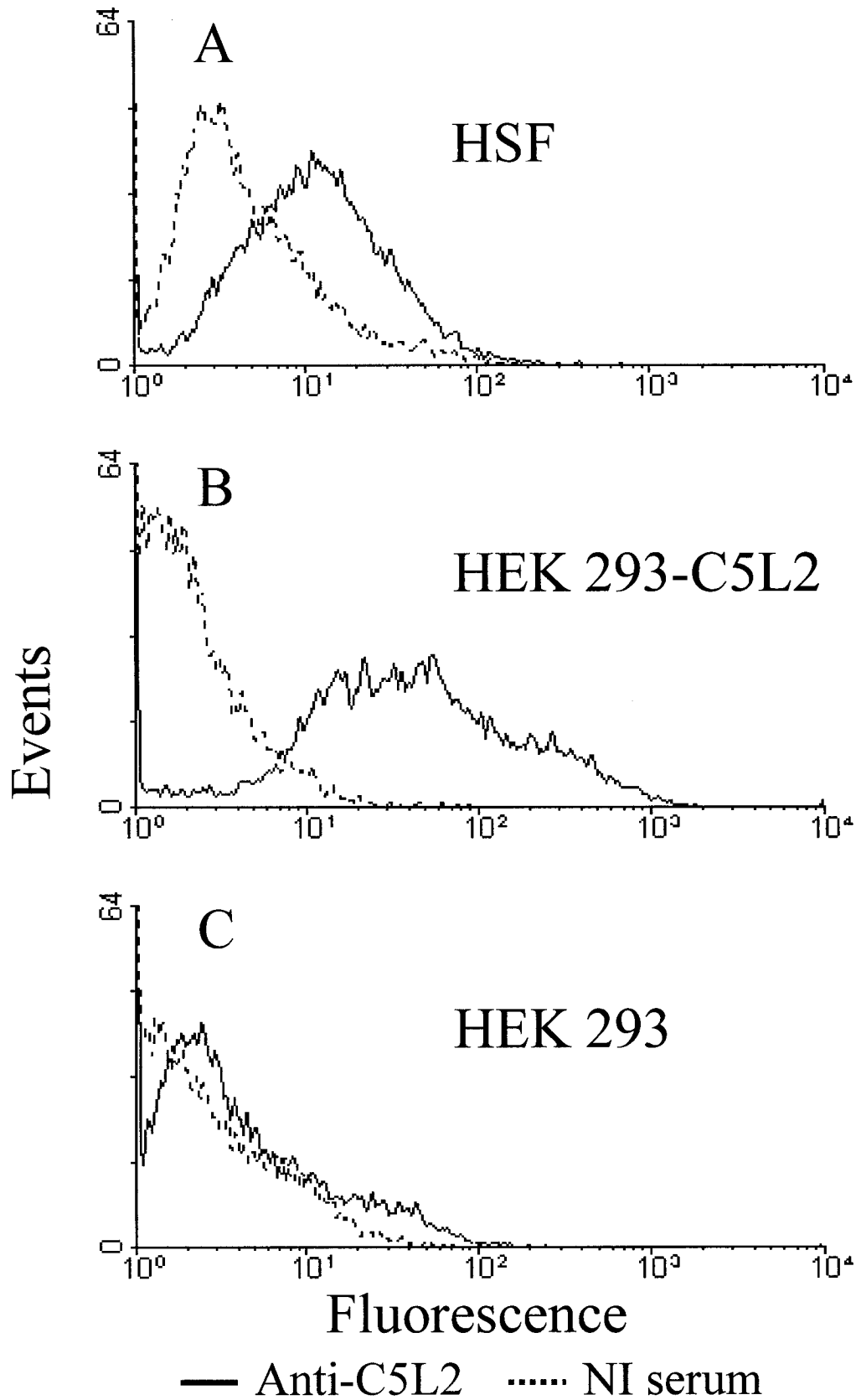

1
2
3
4
5
6
C5L2
GAP
1000
1000

Figure 4.5 Human fibroblasts demonstrate cell surface expression of human C5L2: HSF cells (A), HEK293 cells stably transfected with the C5L2 cDNA (B), and untransfected HEK293 cells (C) were detached nonenzymatically and incubated at 4 °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.


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

PREFACE

Data in the previous chapter showed that although C5a and C5a desArg bind to C5L2, they appear to bind at a site distinct from that for C3a and ASP. Furthermore, ASP neither binds to C5aR or C3aR, nor activates these two receptors. In contrast, ASP binding to C5L2 is high affinity and saturable, similar to the binding of C3a to C3aR. None of the complement fragments activate C5L2 in an assay for anaphylatoxin function. Finally, C5L2 mRNA is expressed in cell types that respond to the TG synthesis stimulatory action of ASP, and C5L2 protein is present on the surface of one of these, human skin fibroblasts. Together these data show that C5L2 is an ASP receptor and could potentially mediate ASP function. In the following chapter, C5L2 is shown to be a functional ASP receptor, by TG synthesis stimulation in cells stably transfected with C5L2, as well as by gene silencing in endogenous cells.

CHAPTER FIVE
C5L2 IS A FUNCTIONAL RECEPTOR FOR ACYLATION STIMULATING PROTEIN

David Kalant[1,2] and Katherine Cianflone[1]

[1]Mike Rosenbloom Laboratory for Cardiovascular Research, Division of Medicine, McGill University Health Centre, Montreal, Quebec, H3A 1A1, Canada; [2]Department of Chemistry/Biochemistry, Concordia University, Montreal, Quebec, H4B 1R6, Canada

(Manuscript in preparation)

Summary

It recently has been demonstrated that the orphan receptor C5L2 binds acylation stimulating protein (ASP) with high affinity, and is expressed in cells that respond to ASP with an increase in triglyceride synthesis (Kalant, D. *et al.* (2003) *J. Biol. Chem.* 278, 11123-11129). Here we show that C5L2 is expressed in both human subcutaneous and omental adipose tissue, in both preadipocytes and adipocytes. Further in mouse tissues, expression is high in adipose tissue compared to other tissues. In gain-of-function assays, stable transfection of the human C5L2 cDNA into normally non-responsive HEK293 cells renders them sensitive to the triglyceride synthesis stimulatory action of ASP (193% ± 33% stimulation at 5 μM ASP, $p<0.001$). Further evidence of ASP activation of C5L2 is demonstrated through the translocation of β-arrestin to the plasma membrane, when HEK293 cells are transfected with the cDNAs for both C5L2 and fluorescently tagged β-arrestin and stimulated by ASP. In loss-of-function assays, when C5L2 expression is downregulated in human skin fibroblasts (HSF) with antisense oligonucleotides, cell surface expression of C5L2 is diminished (down to 54% ± 4% of control, $p<0.001$) and the response to ASP is co-ordinately diminished (down to 64% ± 6% of control, complete inhibition, $p<0.001$). Similarly, in mouse 3T3-L1 preadipocytes, which are responsive to ASP, downregulation of C5L2 with antisense oligonucleotides results in a decrease in C5L2 expression (to 69.5% ± 0.5% of control, $p<0.001$) and a decrease in ASP responsiveness (down to 115% ± 2.5%, $p<0.001$). In both human and murine cells, there is a significant correlation between the extent of down-regulation and

the decreased ASP responsiveness (r^2= 0.579, $p<0.0001$ in HSF and r^2= 0.451, $p<0.05$ in 3T3-L1 cells). This is the first demonstration that C5L2 can mediate the acylation stimulating properties of ASP, and thus provides confirmation that C5L2 is an ASP receptor.

Introduction

The function of ASP as a stimulator of triglyceride synthesis has been well documented in human adipocytes, 3T3-L1 preadipocytes and human skin fibroblasts (HSF) (196,260). The activity of ASP, which is identical to C3a des-Arg^{77}, also is shared by C3a (90). Furthermore, high affinity binding of both ligands to HSF and 3T3-L1 cells (260, Figure 2.3), and of ASP to adipose tissue plasma membranes (111), has been demonstrated. In contrast, no binding of ASP to the C3a receptor (C3aR) has been observed in cells that bind C3a, including U937 macrophage, polymorphonuclear monocytes, transfected RBL cells or transfected HEK293 (257,331, Figure 2.3). Furthermore, ASP does not exhibit the anaphylatoxic functions of C3a: (i) stimulation of eosinophil chemotaxis (332), (ii) prostanoid production by guinea pig macrophages or rat Kupffer cells (333), or (iii) degranulation of U937 cells (257). On the other hand, both ASP (C3a desArg) and C3a have other functions in addition to triglyceride synthesis (TGS) stimulation (335,336). The division between ASP and C3a, in binding and function, may be explained by the existence of two receptors; the C3aR, which binds only C3a, and another that binds both ligands.

We recently have reported that the orphan G protein-coupled receptor, C5L2, binds ASP and C3a with high affinity in transfected RBL cells, and exhibits saturable binding of ASP in transfected HEK293 cells (263). Furthermore, we demonstrated the expression of C5L2 mRNA in adipose tissue, HSF and 3T3-L1 by RT-PCR, and expression of the protein on the surface of HSF by anti-C5L2 polyclonal anti-serum. Although C5L2 also binds C5a and C5a des-Arg^{74}, these

ligands do not stimulate triglyceride synthesis in 3T3-L1 cells. These data suggested that ASP might mediate the triglyceride synthesis stimulating effect of ASP.

In the present study, we provide evidence, through gain-of-function and loss-of-function assays in physiologically relevant cells, that C5L2 not only binds ASP, but also is a functional ASP receptor.

Experimental Procedures

Analysis of Receptor Expression by RT-PCR: Total RNA was isolated by Trizol extraction from freshly isolated samples of tissues. For adipose tissue, mature floating adipocytes and stromal vascular cells (containing preadipocytes) were isolated as described previously (91,197). 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 95 °C, 1 min at 60 °C, 1 min at 72 °C for 35 cycles. Primers for the human C5L2 gene were: sense 5'-CCTGGTGGTCTACGGTTCAG-3' and antisense 5'-GGGCAGGATTTGTGTCTGTT-3'. Primers for the murine C5L2 gene (Ensembl gene ID: ENSMUSG00000041388) were: sense 5'-ATGGCCGACTTGCTTTGT-3' and antisense 5'-CCTTGGTCACCGCACTTTC-3'. Primers for both human and murine 18S rRNA were used as internal controls (Ambion, Austin, TX). Reaction products were separated on a 7.5% polyacrylamide gel and detected by silver

staining (BioRad, Mississauga, ON), and a 100 bp ladder (NEB, Pickering, ON) was used as standard.

Production of ASP and C5L2 Polyclonal Antiserum: ASP was purified from human plasma as described previously (90). Antiserum to human C5L2 was raised in rabbits as previously described (263). Rabbit polyclonal anti-mouse C5L2 was prepared similarly using the carboxyl peptide of mouse C5L2.

Cells and Culture Conditions: HSF were obtained as previously described (339). HSF, 3T3-L1, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 10% (v/v) fetal bovine serum at 37 °C, 5% CO_2. The medium was supplemented with 250 μg/ml G418 for HEK293 stably transfected cells. HEK293 cells stably transfected with the human C5L2 cDNA (HEK-hC5L2) were produced as previously described (263). For all functional assays (β-arrestin, triglyceride synthesis, and cell surface C5L2 assays), cells were transferred to medium without fetal bovine serum two hours prior to the assay.

β-Arrestin Translocation Assay: HEK-hC5L2 cells were seeded onto microscope cover slips placed in 6-well tissue culture plates and transiently transfected with 0.5 μg/well of plasmid containing a cDNA for a fusion protein of β-arrestin and green fluorescent protein (GFP) (350) (gift from Stephane Laporte). Two days later, cells were preincubated in serum free medium for 2 hours, then incubated for indicated times in serum-free medium with 10 μM ASP. Cells were fixed with 4% paraformaldehyde and photographed under a fluorescent microscope as described previously (350).

Triglyceride Synthesis Assay: Triglyceride synthesis assays were performed as previously described (90). Briefly, cells were preincubated in serum free medium for 2 hours, then cells were incubated for 4 hours at 37 °C with vehicle (PBS), ASP or insulin (Sigma, St Louis, MO) at the concentrations indicated, in the presence of 100 μM ^{3}H-oleate complexed to albumin (molar ratio 5:1) diluted in serum-free medium. Cells were washed with PBS, and lipids extracted for separation by thin layer chromatography. Cell protein was dissolved in 0.1 N NaOH, and aliquots taken to measure cell protein. Triglyceride synthesis was measured as pmol ^{3}H-oleate incorporated into triglyceride per mg soluble cell protein.

Cell Surface C5L2 Quantitation Assay: Following incubation in serum free medium for 2 hours, HSF and 3T3-L1 cells were incubated with anti-human or anti-mouse C5L2 antiserum, respectively, at 1/100 dilution in buffer containing hepes, albumin, glucose, calcium and magnesium, pH 7.2 (HAG-CM), for 30 minutes at room temperature. After two rinses with HAG-CM, cells were incubated with ^{125}I-protein A (ICN, Costa Mesa, CA) at 1/1000 dilution in HAG-CM for 30 minutes at room temperature. After a further two rinses with HAG-CM, cell protein was dissolved in 0.1 N NaOH for one hour, and aliquots were taken for γcounting and cell protein determination.

Design and Transfection of Antisense Oligonucleotides: One DNA oligonucleotide was made to the translation start site of each of the human and mouse C5L2 mRNAs. Two other oligonucleotides were designed with the Primer3 program at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi,

using the same default criteria as that for PCR primers. These target regions in the middle and the last 25% of the translated sequence. Oligonucleotides were all-phosporothioated for stability. Oligonucleotides for the human C5L2 mRNA were: 5'-GCTGACAGAATCGTTCCCCAT-3' (1), 5'-CTGAACCGTAGACCACCAGG-3' (2), and 5'-ACAGGAAGAGCATGGGATTG-3 (3)'. Oligonucleotides for the mouse C5L2 mRNA were: 5'-CTGGTGGTGTGGTTCATCAT-3' (1), 5'-TAGGAAGAGGTCTCCGCTGA-3' (2), and 5'-CAAATGAAAAACCCCACCAC-3' (3). The reverse complement of oligonucleotide 2 was used as a control oligonucleotide for each C5L2 mRNA, with some manual alterations necessary for the human sequence so that it would not match any sequence in the human genome. The oligonucleotides were: human, 5'-ATCTTAACTAGCTAGCAATC-3' (Ctl), and mouse, 5'-TCAGCGGAGACCTCTTCCTA-3' (CTL). Two sets of either HSF or 3T3-L1 cells, at 40 to 60% confluency in 24-well plates, were transfected with Oligofectamine (Invitrogen, Burlington, ON) according to the manufacturer's instructions, using 2.5 (A, C) or 4 (B, D) μl of a stock of 20 μM oligonucleotide combined with 1 (A, B) or 2 (C, D) μl of Oligofectamine, in triplicate. Cells were assayed 2 days post transfection in parallel for (i) quantitation of C5L2 by Protein A immunoassay and (ii) ASP stimulation of triglyceride synthesis.

Results and Discussion

C5L2 mRNA is Expressed at High Levels in Tissues that Are Responsive to ASP: We have shown recently that C5L2 mRNA is expressed in human adipose

tissue, HSF, and 3T3-L1 preadipocytes (263), which all respond to ASP with an increase in TGS (263) and glucose transport (92,351). Previous reports have shown the relative expression in a number of tissues (324,325), but none have included adipose tissue. We report for the first time a comparison of C5L2 mRNA expression in adipose with other tissues from mouse, using semi-quantitative RT-PCR with primers to mouse C5L2 mRNA. PCR products were electrophoresed on a polyacrylamide gel, quantified by densitometry, and compared to a standard curve made with the known masses of the DNA ladder. Average values for the tissues examined are shown in Figure 5.1. C5L2 mRNA is expressed at the highest levels in intra-abdominal fat depots: omental, perirenal and gonadal adipose tissue, with somewhat lower levels in brown adipose tissue (BAT), pectoral and inguinal fat. This is consistent with previous reports that adipose tissue is the most sensitive to ASP stimulation of TGS (91).

RNA also was isolated from human subcutaneous and omental adipose tissue. In addition, in some preparations, adipose tissue was separated into stromal vascular cells (which contain the preadipocytes) and mature floating adipocytes. RT-PCR with primers for human C5L2 mRNA indicates the expression of C5L2 mRNA in preadipocytes and mature adipocytes from both subcutaneous and omental depots (Figure 5.2).

Gain-of-Function Activation of C5L2 by ASP: We have shown previously that ASP binds to C5L2 with high affinity (263). Here we show that ASP activates C5L2 in HEK-hC5L2 cells. β-arrestin is a cytosolic protein that mediates the internalization and desensitization of G protein-coupled receptors (GPCR). It has

been found to translocate from the cytosol to the plasma membrane and associate only with GPCR, and only once the receptors have been activated by their ligands (350,352). HEK293 and HEK-hC5L2 cells, transiently transfected with a cDNA coding for a β-arrestin/GFP fusion protein were incubated with or without ASP for indicated times. Fluorescent micrographs show a diffuse distribution of β-arrestin in HEK293 cells incubated with or without ASP (data not shown). The same diffuse pattern is seen in HEK-hC5L2 cells without ASP (Figure 5.3, 0 min), whereas a clearly punctate distribution is seen along the membrane of HEK-hC5L2 cells when incubated with ASP for 2.5 or 5 minutes (Figure 5.3). This distribution may be attributed to the association of β-arrestin with C5L2 in clathrin-coated pits and vesicles. To date, no evidence has been found that β-arrestin is recruited to GPCR at the plasma membrane without agonist activation, thus these data clearly indicate C5L2 activation by ASP.

By 15 minutes, the fluorescent spots are seen throughout the cell, and no longer at the plasma membrane, indicating the internalization of β-arrestin with endocytic vesicles. At 20 and 40 minutes the fluorescence is associated with fewer, larger vesicles. These vesicles are possibly lysosomes, suggesting that C5L2 may be preferentially degraded rather than recycled to the plasma membrane.

Gain-of-Function Triglyceride Synthesis Stimulation by ASP in HEK-hC5L2 Cells: We have reported previously that HEK293 cells are competent for triglyceride synthesis (TGS), but ASP does not stimulate TGS in these cells (263). However, when HEK-hC5L2 cells were incubated with increasing

concentrations of ASP, TGS was stimulated several fold, and was maximal at 5 μM ASP (193% ± 33% stimulation, $p<0.001$). By contrast, TGS in HEK293 was not stimulated even at 10 μM ASP (Figure 5.4, top panel). The effect in HEK-hC5L2 cells is comparable to the stimulation seen in HSF and 3T3 cells (263). On the other hand insulin, another known stimulator of TGS, which increases TGS in HSF cells (263), has no effect on either HEK293 or HEK-hC5L2 cells even at a high concentration of 100 nM (Figure 5.4, bottom panel). This is the first demonstration that C5L2 can mediate TGS stimulation by ASP. As insulin does not stimulate TGS in HEK-hC5L2 cells , the mediation appears to be specific for ASP. Although an over-expressing cell might be expected to respond to a higher degree than endogenous cells, it is likely that the intracellular signalling pathway is not optimal, and is expressed at a lower level in the HEK293 cells and is therefore limiting. The activation and TGS stimulation data are in contrast, but not contradictory, to those recently reported by Okinaga *et al.* (353) showing that C5L2 is not activated by C5a. Furthermore, we previously have demonstrated that C5a, as well as C4a and the desArg counterparts, are all unable to stimulate triglyceride synthesis in 3T3-L1 cells (263).

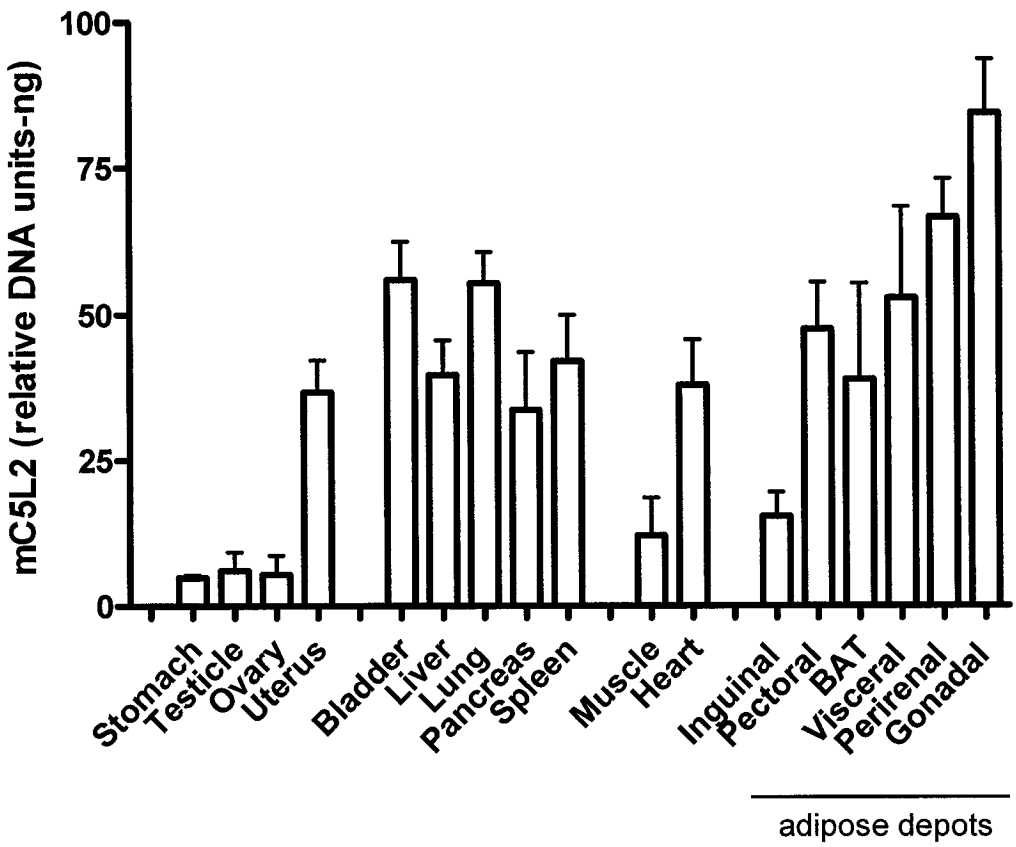
Loss-of-Function by Down-Regulation of C5L2 mRNA Diminishes both C5L2 Expression and TGS Stimulation by ASP: Because the above results clearly show that ASP can function through C5L2 in transfected cells, we were interested in assessing the role of C5L2 in cells that normally respond to ASP. A gene-silencing technique was employed to specifically inhibit translation of the C5L2 mRNA. The antisense technique involves the transfection of DNA

oligomers that are complementary to the target mRNA and, upon binding, inhibit passage of the mRNA through the ribosome (354). Generally, oligonucleotides are designed to different regions of the mRNA of interest, and the most effective determined empirically. Two amounts of three antisense oligonucleotides directed to human C5L2, either alone or in combination, were transfected into HSF cells using two amounts of transfection reagent. A non-silencing oligonucleotide was used as a control. After two days, identical sets of cells were assayed for either TGS, or C5L2 expression. C5L2 expression in HSF was decreased to 54% ± 4% ($p<0.001$) relative to the untreated cells (= 100% expression) (Figure 5.5, top panel) depending on the conditions. TGS stimulation by ASP in control oligonucleotide treated cells was comparable to ASP stimulation in untreated cells (144% ± 5%). However treatment with anti-sense decreased the ASP stimulation down to 64% ± 6%, $p<0.001$ vs. no treatment (=100%). In some cases, the suppression of ASP stimulation resulted in TGS at or lower than basal levels (set at 100%) of TGS (complete inhibition of ASP stimulation). Overall, there was a close correlation between downregulation of C5L2 and the decrease in ASP responsiveness, as shown in Figure 5.5, bottom panel ($r^2 = 0.579$ and $p < 0.0001$).

Antisense experiments also were carried out in mouse 3T3-L1 preadipocytes with oligonucleotides directed to mC5L2. As shown in Figure 5.6, top panel, depending on the amount and combination of antisense oligonucleotides there were decreases in C5L2 expression down to 69.5% ± 0.5% ($p< 0.001$ vs. untreated cells). On average, non-immune serum background represented 50%

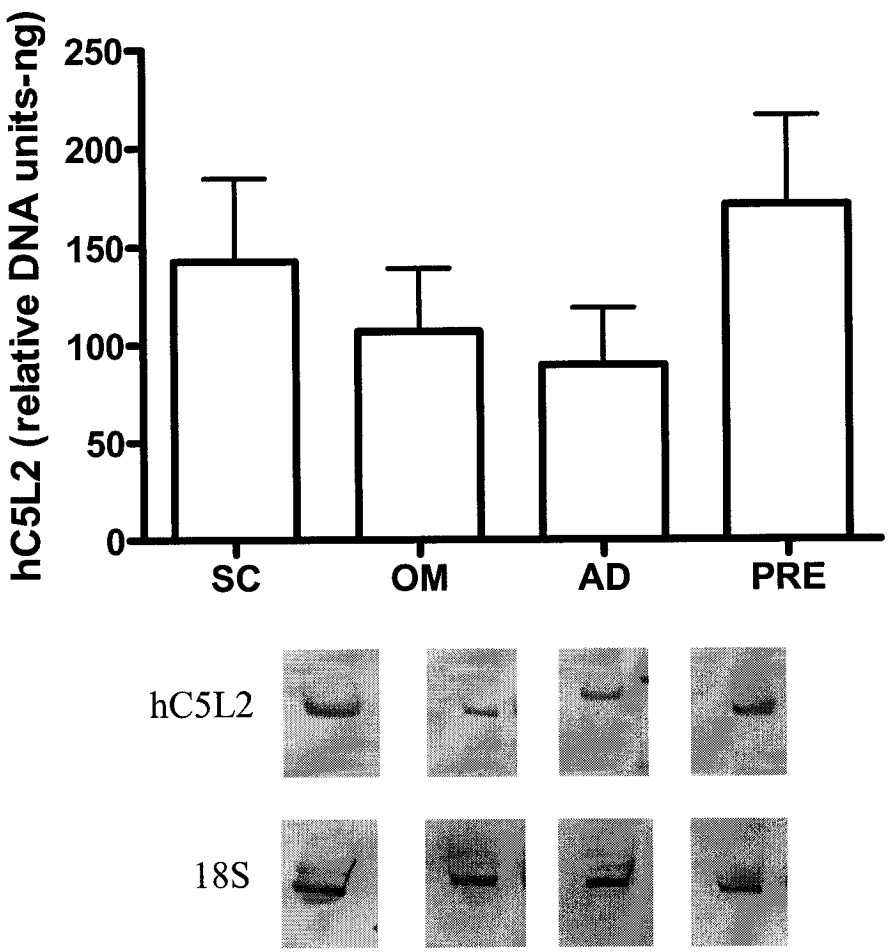
of the amount of the ^{125}I protein A bound. As with HSF, treatment with control oligonucleotide did not prevent the ASP stimulation of TGS in 3T3-L1 cells (242% ± 9% for control oligonucleotide vs. 177% ± 22.6% no treatment). However treatment with antisense resulted in a decrease in ASP stimulation of TGS, which again correlated with the decrease in C5L2 cell surface expression (Figure 5.6, bottom panel, $r^2 = 0.451$ and $p < 0.05$). The fact that the same oligonucleotide conditions that lower C5L2 expression, also lower the ability of ASP to stimulate TGS, confirms the role of C5L2 as a mediator of ASP function in endogenous cells.

Figure 5.1 C5L2 mRNA is expressed at high levels in adipose tissue



Total RNA, isolated from mouse tissues by Trizol extraction, was used for semi-quantitative RT-PCR with primers sets for mouse C5L2 mRNA, and 18S rRNA as internal standard. Products were electrophoresed on a polyacrylamide gel and detected by silver staining. Bands were quantified by densitometry and compared to a standard curve made from the known masses of a 100 bp ladder.

Figure 5.2 C5L2 is expressed in human subcutaneous and omental adipose tissue, and in both preadipocytes and mature adipocytes



Total RNA was isolated directly from human adipose tissue, or from preadipocytes and mature adipocytes isolated from the tissue. RT-PCR was performed with primers for human C5L2 mRNA and for 18S rRNA as internal standard, then quantified by densitometry. Bands for C5L2 were normalized to 18S.

Figure 5.3 HEK-hC5L2 cells expressing fluorescently tagged β-arrestin are activated by ASP: HEK-hC5L2 cells were transiently transfected with a plasmid containing the cDNA for a fusion protein of β-arrestin and green fluorescent protein. Two days later, the cells were incubated with 10 μM ASP for the indicated times, then fixed and assayed for fluorescent distribution. At 0 minutes the fluorescence is homogeneously distributed throughout the cytosol (basal, non-activated state). At 2.5 and 5 minutes the fluorescence shows a punctate pattern at the plasma membrane, indicative of β-arrestin translocation to the membrane and association with C5L2. By 15 minutes, the punctate pattern is seen through the cell, indicating internalization with endocytic vesicles. At 20 and 40 minutes, there are larger green spots, possibly indicating association with lysosomes. With or without ASP, only a homogeneous distribution of fluorescence is seen in native HEK293 cells transfected with the β-arrestin cDNA (data not shown).

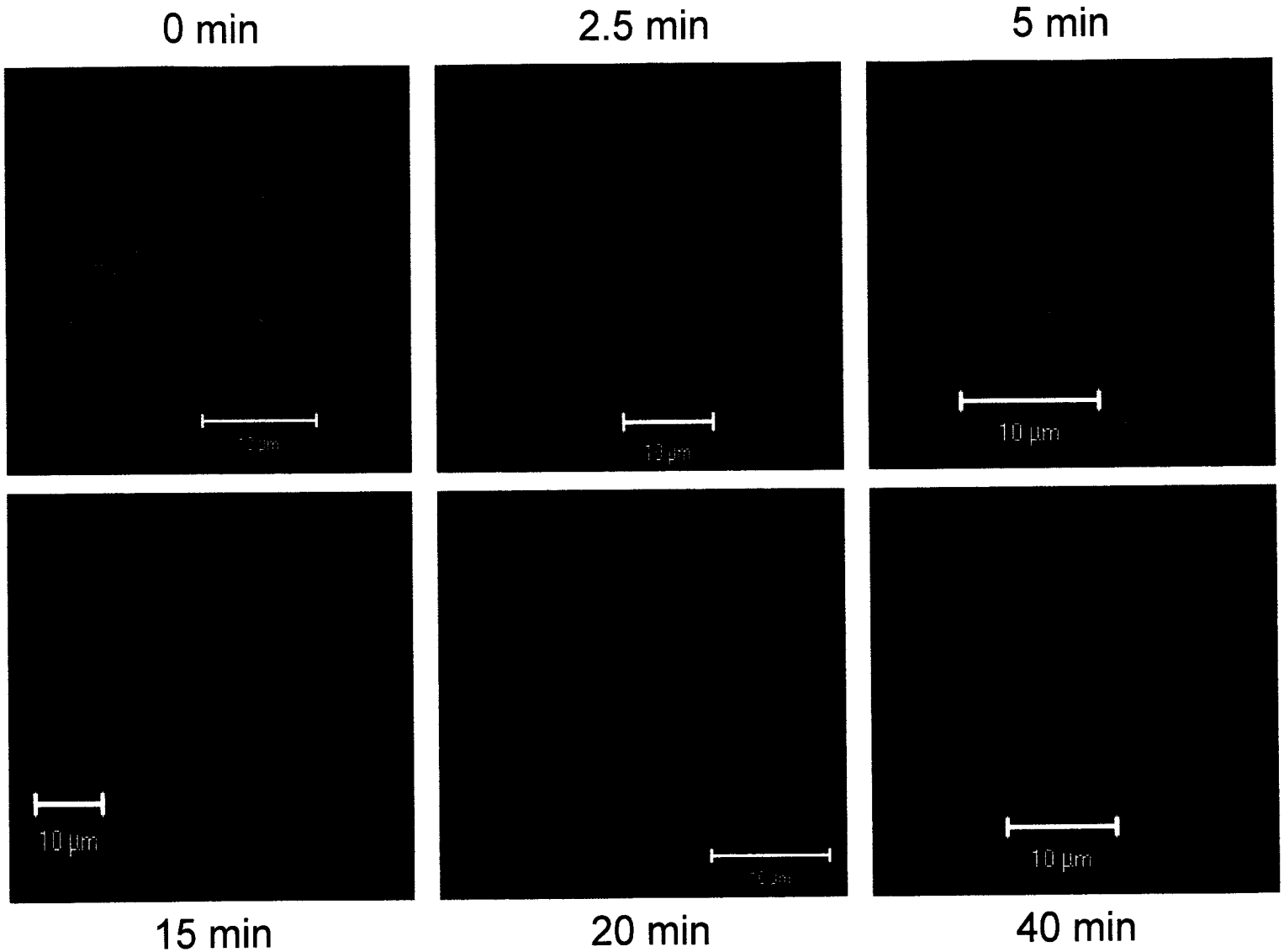

0 min
2.5 min
5 min
10 µm
10 µm
10 µm
10 µm
10 µm
15 min
20 min
40 min

Figure 5.4 ASP, but not insulin, stimulates TG synthesis in HEK-hC5L2 cells: HEK293 and HEK-hC5L2 cells were incubated with increasing concentrations of ASP (top panel) or insulin (bottom panel) as indicated, in the presence of ^{3}H-oleate. After 4 hours, lipids were extracted, separated by thin layer chromatography, and the incorporation of ^{3}H-oleate determined. Triglyceride synthesis was measured as pmol ^{3}H-oleate incorporated into triglyceride per mg soluble cell protein, with basal set at 100%. The data shown is the average of five experiments. By ANOVA, only the HEK-hC5L2 with ASP show a significant change in TG synthesis over time ($p < 0.05$). By Bonferoni post-hoc test, the indicated points are significantly different from basal (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

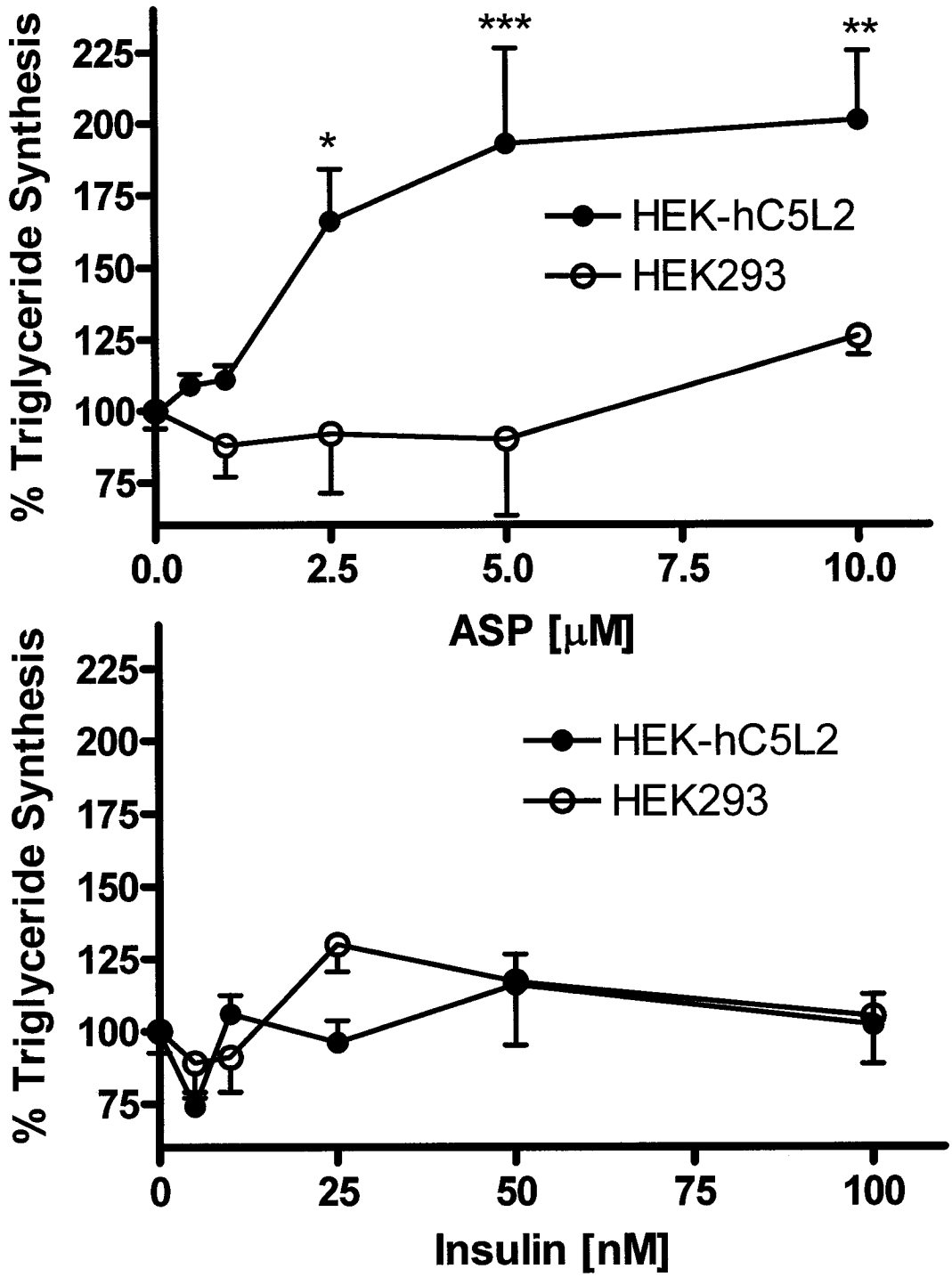
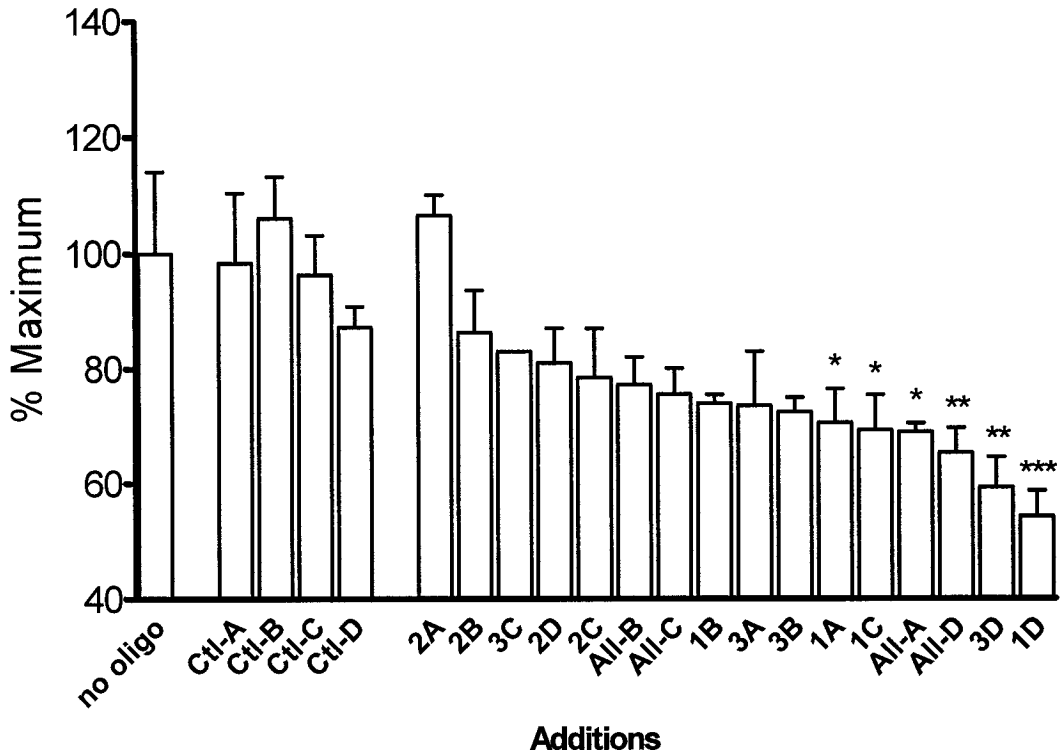

% Triglyceride Synthesis
225
200
175
150
125
100
75
*
***
**
HEK-hC5L2
HEK293
0.0
2.5
5.0
7.5
10.0
ASP [μM]
% Triglyceride Synthesis
225
200
175
150
125
100
75
HEK-hC5L2
HEK293
0
25
50
75
100
Insulin [nM]

Figure 5.5 Antisense oligonucleotides to human C5L2 mRNA diminish both C5L2 expression and TGS stimulation by ASP: Antisense oligonucleotides 1, 2 or 3 alone, or combined (All), or control oligonucleotide (Ctl), at conditions A, B, C or D (see methods), were transfected into HSF cells seeded the day before in 24 well plates. Two days post-transfection cells were assayed for C5L2 expression by antiserum to C5L2 and ^{125}I-protein A (top panel). The results are compared to C5L2 expression in untransfected cells (no oligo), which are set at 100%. There was a significant drop in C5L2 expression as assessed by ANOVA ($p < 0.001$). By Bonferoni post-hoc test, the indicated conditions are significantly different from untransfected cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). An identical set of cells was assayed for ASP stimulation of TGS. The bottom panel shows the correlation ($r^2 = 0.579$ and $p < 0.0001$) between C5L2 expression and TGS stimulation by ASP, where no stimulation (basal) = 100% TGS, and ASP stimulation (no oligo) = 145% TGS.


140
120
100
80
60
40
% Maximum
no oligo
Ctl-A
Ctl-B
Ctl-C
Ctl-D
2A
2B
3C
2D
2C
All-B
All-C
1B
3A
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1A
1C
All-A
All-D
3D
1D
*
*
*
**
**
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Additions

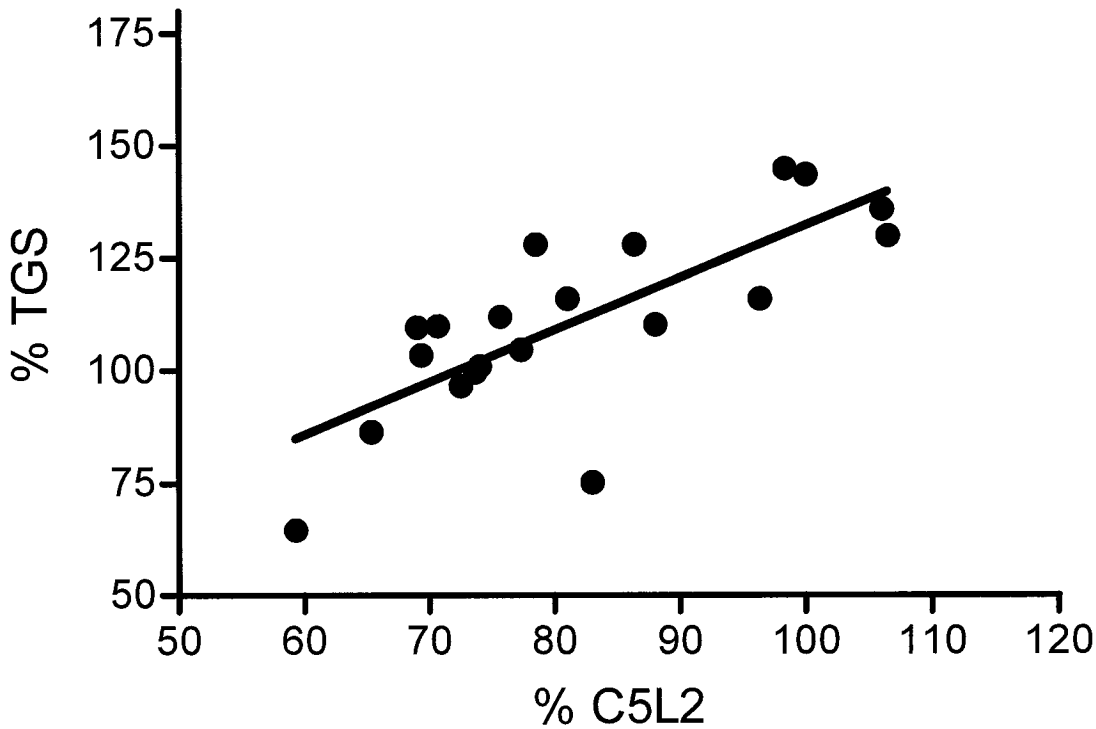
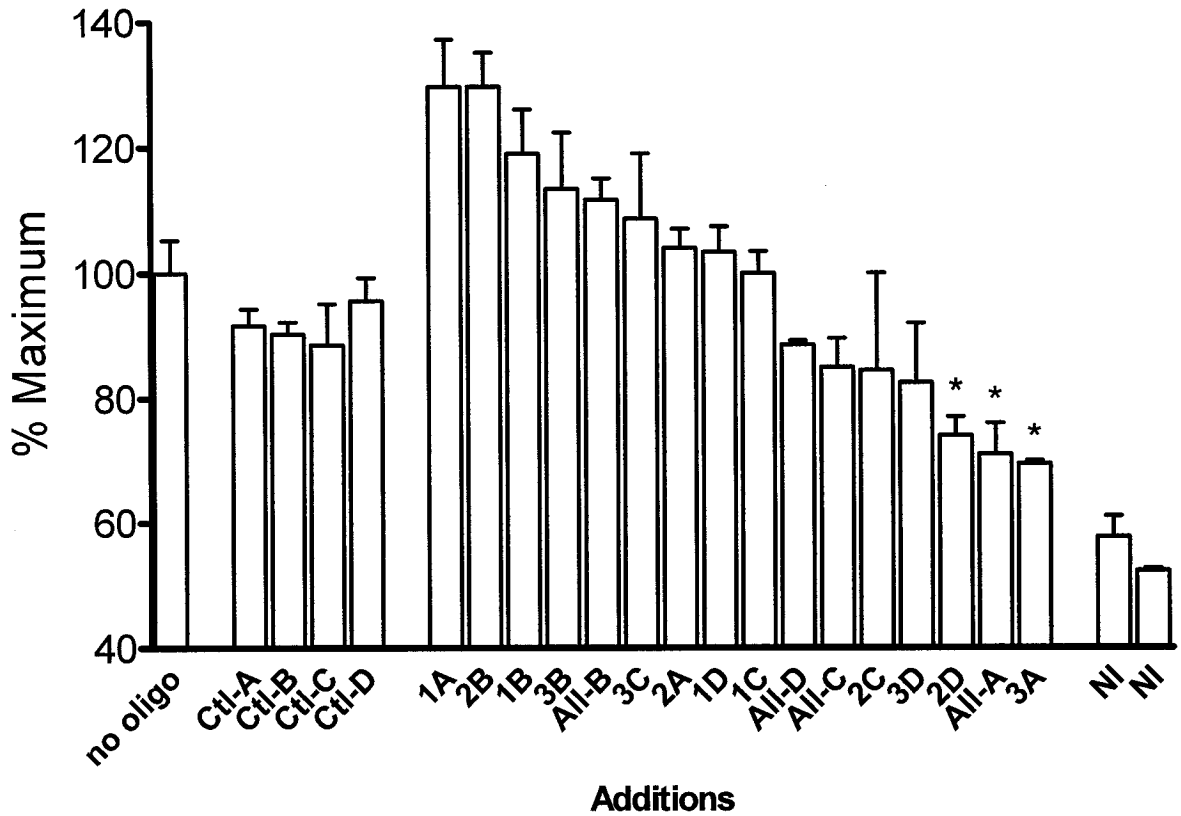
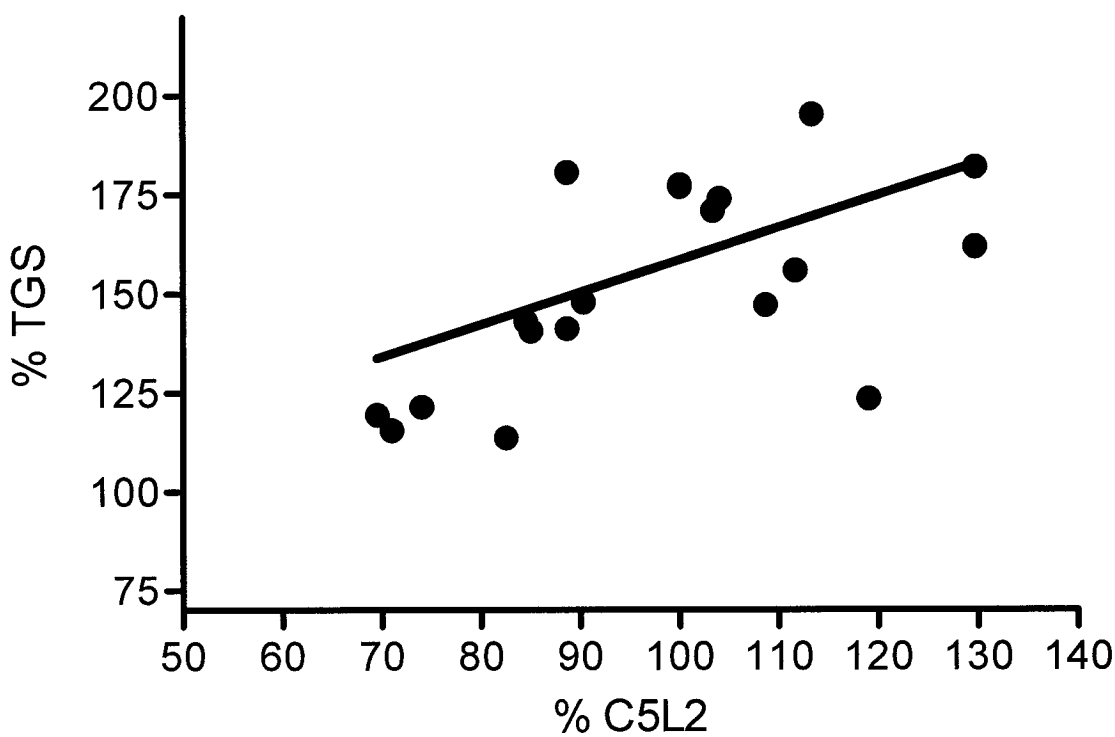

175
150
125
100
75
50
% TGS
50
60
70
80
90
100
110
120
% C5L2

Figure 5.6 Antisense oligonucleotides to mouse C5L2 mRNA diminish both C5L2 expression and TGS stimulation by ASP: Antisense oligonucleotides 1, 2 or 3 alone, or combined (All), or control oligonucleotide (Ctl), at conditions A, B, C or D (see methods), were transfected into 3T3-L1 cells seeded the day before in 24 well plates. Two days post-transfection cells were assayed for C5L2 expression by antiserum to C5L2 and ^{125}I-protein A (top panel). Non-immune serum (NI) indicates background binding of ^{125}I-protein A. The results are compared to C5L2 expression in untransfected cells (no oligo), which are set at 100%. There was a significant drop in C5L2 expression as assessed by ANOVA ($p < 0.001$). By Bonferoni post-hoc test, the indicated conditions are significantly different from untransfected cells ($*p < 0.05$). An identical set of cells was assayed for ASP stimulation of TGS. The bottom panel shows the correlation ($r^2 = 0.451$ and $p < 0.05$) between C5L2 expression and TGS stimulation by ASP, where no stimulation (basal) = 100% TGS, and ASP stimulation (no oligo) = 177% TGS.


140
120
100
80
60
40
% Maximum
no oligo
Ctl-A
Ctl-B
Ctl-C
Ctl-D
1A
2B
1B
3B
All-B
3C
2A
1D
1C
All-D
All-C
2C
3D
2D
All-A
3A
NI
NI
*
*
*
Additions


200
175
150
125
100
75
% TGS
50
60
70
80
90
100
110
120
130
140
% C5L2

CHAPTER SIX
DISCUSSION

Summary

Over the last 15 years, both *in vitro* and *in vivo* studies have indicated a physiologically important role for ASP in the uptake and storage of dietary fat, primarily in adipose tissue. Studies in cultured cells have indicated a link between ASP receptor levels and certain dyslipidemias, including hyperapoB. They have also elucidated differences between different adipose tissue depots in their response to ASP. Consistent with the closer association of omental adipose tissue to diabetes and cardiovascular disease, ASP is less potent at stimulating fatty acid storage in omental adipose tissue as compared to subcutaneous tissue.

Studies in human subjects and mice, including C3 knockout, ASP deficient, mice, have pointed to a role for ASP in the development of obesity. On one hand, obese subjects show increased post-prandial ASP production and tissue fatty acid uptake compared to the non-obese. On the other hand, post-obese women have lower ASP than those who were never obese, suggesting an increased sensitivity to ASP. Both results imply a role for ASP in a possible genetic predisposition to obesity. Consistent with the above, ASP deficient mice appear to be protected against the development of obesity.

Taken together, the *in vitro* and *in vivo* studies supported the merit of further investigation into the mechanism of ASP function. The rapid world-wide increase of obesity, and the associated diabetes and cardiovascular disease risk, make

clear the need for research that may lead both to an increased understanding of the development of these diseases, as well as possible treatment.

As reviewed in chapter 2, the specific ASP actions and binding patterns on cultured cells strongly suggested the existence of an ASP receptor. The evidence that this receptor is distinct from the C3aR, has been provided by reports from many labs including our own. These reports consistently show that ASP neither binds to the C3a receptor, nor induces the immunological responses attributable to C3a, a protein that differs by only one amino acid. Therefore, a search for the ASP receptor was initiated. As detailed in chapter 3, three strategies to isolate an ASP receptor were employed. These included screening of an adipose cDNA library, either by gene expression and binding of ASP, or gene screening with probes based on GPCRs assumed to be homologous to the ASP receptor (C3aR, C5aR and the fMLP receptors). When these two methods failed to isolate a receptor, consensus sequences of the above receptors were used to search databases for an orphan gene that might be an ASP receptor. The testing of several possible candidates revealed that none bound ASP above a background level.

During the time that the three strategies were undertaken, several techniques were refined and improved, such that another attempt at the original library screening was thought to be warranted. This second attempt did indeed produce plasmid pools enriched in ASP-binding clones. Although the original strategy was now working, the testing of C5L2, an orphan receptor, showed that it bound ASP with high affinity. The library was put aside, and C5L2 was investigated further.

As the data reported in chapters 4 and 5 reveal, C5L2 not only binds ASP, but is activated by ASP, and mediates the triglyceride synthesis stimulatory action of ASP. This was shown through a gain-of-function assay in HEK293 cells stably transfected with C5L2, as well as a loss-of-function assay in normally responsive cells when C5L2 expression was down-regulated. Consistent with these data, RT-PCR of mouse tissues showed that C5L2 is expressed in tissues and cells that respond to ASP, with particularly high expression in many adipose depots. As well, C5L2 is expressed in sub-cutaneous and omental human adipose tissue, in both preadipocytes and mature adipocytes.

Two points should be mentioned concerning the cloning strategies used. First, as mentioned in chapter 3, C5L2 was cloned by two independent labs one year after our search for orphan receptors (324,325) . An alignment of the C5L2, C3aR and C5aR amino acid sequences reveals that C5L2 has approximately 47% and 53% homology with C3aR and C5aR, respectively (Figure 6.1). Since one of the investigators cloned C5L2 by homology to C5aR, it is quite likely that our search for orphans would have identified C5L2 had its sequence been known at the time.

Second, recent knowledge gained from the several hundred GPCRs that now have been cloned suggests that there are several conserved residues in transmembrane (TM) 2 of homologous GPCRs (355), and this is the case for C5L2, C3aR and C5aR (Figure 6.1). The alignment also shows that TM3 is fairly highly conserved among these three receptors (Figure 6.1). It is tempting to speculate that had these two TM regions, rather than TM1, 4 and 7, been chosen

to design the degenerate primers used to make the PCR probes, the second strategy might have been successful.

G Protein Coupled Receptors

There are three superfamilies of cell surface signaling receptor, based on their signaling method and transmembrane topology; i) receptors with intrinsic tyrosine kinase activity, such as the insulin receptor, ii) receptors directly linked to tyrosine kinases, but without intrinsic activity, such as those for the interleukins, iii) receptors that couple to heterotrimeric GTP-binding proteins (GPCR). The first two families generally have single TM domains, while the GPCRs have 7 TM regions. The vast majority of receptors belong to the GPCR superfamily, with almost 1000 members, including orphans, sequenced to date. Included in the GPCR superfamily are receptors for hormones, neurotransmitters, chemokines, biogenic amines, ions, and sensory receptors for odorants, taste and light (356,357). The GPCRs are, in turn, divided into 3 main classes, A, B and C, on the basis of sequence similarity. Class B (secretin-like) and Class C (metabotropic glutamate-like) each contain relatively few members. The largest group by far is class A, characterized by homology with the photon receptor rhodopsin, and within this class the chemokines represent the largest subfamily. Chemokines are a group of small (8-14 kDa) basic proteins which, despite sequence differences, generally have a similar three-dimensional structure (357). The amino acid sequence of C5L2 was submitted to a GPCR classification web

service (358), and determined to be a member of Class A, as are C3aR and C5aR.

The generally accepted paradigm for GPCR signaling involves; 1) binding of a ligand to the GPCR to cause a conformational change, 2) activation of an associated GTP-binding protein (G protein) which then activates downstream signaling molecules, 3) desensitization of the receptor via phosphorylation by GPCR kinases (GRK), 4) coupling of β-arrestin to initiate internalization, and 5) degradation or recycling to the cell surface. Although the molecular determinants for C5L2 interaction with ASP, G proteins, GRK and β-arrestin are not yet known, comparison with other GPCRs which have been more thoroughly studied, including the related C3aR and C5aR, yield some predictions and point to future research.

Ligand Binding to GPCRs

Although C3a and ASP differ only by the presence or lack of the C terminal arginine, only C3a binds to C3aR, while both ligands bind to C5L2. However this binding pattern is not unique to C3a/ASP. Bradykinin (BK), a nine amino acid peptide with effects that include vasodilation, hyperalgesia, and bronchoconstriction (359-361) exists in both C-terminal Arg and desArg forms. These two forms differentially stimulate the G protein-coupled BK receptors B1 and B2. B1 is activated to a greater degree by BK desArg than by BK, while B2 is stimulated with much greater potency by BK compared to BK desArg (361). Furthermore, a recently discovered BK receptor, GPR100, is activated by BK but

not at all by BK desArg (362). The examples of BK/ BK desArg and C3a/ASP clearly indicate the possible effects of the presence or absence of one critical amino acid.

The ligand binding sites in the GPCRs for small molecules, such as the biogenic amines, involve specific amino acids in a binding pocket formed by the TM domains (321,363). Binding of a ligand to this pocket has been shown to alter the positions of the TM helices, relative to each other, leading to a conformational state which is competent to activate G proteins (364,365). Although the TM binding pocket is also generally required for larger peptides and proteins, important binding sites exist in the second extracellular (e2) loop, and often in the N terminal domain as well. This has been shown for the angiotensin, endothelin and neuropeptide Y receptors, among others (363). For some of the largest ligands, an extended N terminus appears to be sufficient for binding (321).

Mutation of three charged amino acids in the TM domains of C3aR abolished C3a binding, while mutation of two others demonstrated both decreased binding and function, indicating the importance of these residues in C3a binding (366). As indicated in Figure 6.1, four of the five amino acids, including the three critical amino acids, are conserved in C5L2, where they might potentially serve the same function. Only one of the mutated TM amino acids is negatively charged, and perhaps interacts with the C3a C-terminal arginine, which is required for binding to C3aR. An acidic amino acid is conserved in this region of C5L2, although an arginine is not required for binding in this case. Thus the question remains as to why there is differential binding of C3a and ASP to C5L2 and

C3aR. Studies such as this should be interpreted with caution however, since these TM amino acids may be important in maintaining a conformation that allows C3a to bind, rather than being involved in a direct interaction with C3a. Indeed, in the C-terminal peptide of C3a that is competent for C3aR activation, there are no negatively charged amino acids that would be expected to interact with the basic amino acid of the C3aR binding pocket.

C3aR has an unusually long e2 loop (255), containing clusters of aspartic acids at the N-terminal and C-terminal ends of the loop. Although most of the loop could be deleted without altering C3a binding, mutagenesis of the aspartic acids caused a decrease in C3a affinity (367). Conversely, deletion of the relatively short N-terminal tail of C3aR had no effect on C3a binding (322,367) Together, the mutagenesis studies suggest that the TM regions of C3aR are indispensable, the e2 loop contributes, but the N terminal tail plays no role in C3a binding. Based on the above data, another model for C3a binding to C3aR can be proposed. Perhaps the C-terminal arginine of C3a interacts with one of the aspartate clusters in the e2 loop, and the C-terminal tail of C3a enters the TM binding pocket as a loop. C5L2 has only three negatively charged amino acids in the e2 loop, and perhaps does not require interaction at this site. This may be the reason for the lack of distinction between C3a and the desArg version, ASP.

In comparison, while TM regions and possibly the e2 loop are important for binding of C5a to C5aR (321,322,367), deletion of the N-terminal tail of C5aR demonstrated that this sequence is also required (321,367). As shown in Figure 6.1, the e2 loop contains five acidic amino acids that may be involved in C5a

binding. The N terminus contains seven aspartic acids which are suggested to be involved in ligand binding (321), whereas C3aR contains only two negatively charged amino acids in this region.

The extracellular regions of C5L2 more closely correspond to those of C5aR than of C3aR. Although C5L2 has only three negatively charged amino acids in the e2 loop (which perhaps are less important than in C3aR and C5aR), it has eight in the N terminus (Figure 6.1), suggesting that, as for C5aR, the N terminus plays an important role in ASP binding. The different binding models for C5L2 and C5aR, compared to C3aR, are consistent with the known activities of the native ligands and their respective C-terminal peptides. While a C-terminal peptide of C3a can activate C3aR almost as well as native C3a (367), although at much higher concentrations, only full length ASP and C5a can stimulate C5L2 (260) and C5aR (367), respectively. The requirement of full length ASP or C3a for stimulating TG synthesis has also been demonstrated by altering the ASP core, either by reducing the disulphide bonds that stabilize it, or by chemically modifying the positively charged amino acids. In both cases TG synthesis was markedly reduced compared to native ASP (260). Whether or not the implicated residues in C5L2 are truly involved in ASP binding and activation, can likely be answered by studies such as those carried out on C3aR and C5aR.

The fact that many GPCRs are activated by ligand binding to both extracellular and TM regions, has led to a proposed model for binding of angiotensin II to the AT_1 receptor (364). In this model initial ligand binding to extracellular regions causes a conformational shift in some of the TM domains,

allowing progressively deeper entry into the TM binding pocket. Thus the receptor passes through several states of intermediary activity, from inactive to fully active.

G Protein Coupling and Signaling

The heterotrimeric G proteins are comprised of α, β and γ subunits. Upon binding of a ligand to a GPCR, the α subunit exchanges the bound GDP for GTP, and dissociates from the $\beta\gamma$ subunits, which stay tightly bound. While it is the GTP-bound α subunit that most commonly activates downstream signaling pathways, the $\beta\gamma$ complex has also been shown to activate ion channels (368) and phosphoinositol-3 kinase (369). Hydrolysis of the GTP by the intrinsic GTPase activity of the α subunit returns the α subunit to an inactive state and promotes reassociation with the $\beta\gamma$ complex.

There are at least 18 subtypes of α subunits, divided into four subfamilies; G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$. Five subtypes of β subunits and eight subtypes of γ subunits have been identified, and these, in combination with the many different α subunits, provides a wide range of signaling pathways for GPCRs. Most GPCRs couple to only one Gα subtype, but several couple to more than one, and a few, such as the TSH receptor, couple to all four (368). Depending on the type(s) and number of G proteins activated, one or several signaling pathways can be activated. The particular G protein to which a GPCR couples may also change depending on the cell type, leading to different functions in different cells.

C3a, for example, couples to $G_{i/o}$ in leukocytes, but appears to act through $G_{12/13}$ in endothelial cells (370).

Through the use of specific inhibitors, the involvement of G_s and $G_{i/o}$ in ASP function have been ruled out (262, manuscript in preparation) The most likely candidate is $G_{q/11}$, which is known to activate phospholipase Cβ (PLCβ) (368). It has been shown that ASP stimulation of HSF results in a rapid increase in intracellular diacylglycerol, implicating PLCβ (262). DAG induces the translocation of protein kinase C (PKC) from the cytosol to the plasma membrane, where PKC is active. It has been demonstrated that the translocation of PKC can be stimulated by ASP (262).

Several intracellular regions of GPCRs have been implicated in G protein coupling and activation. Both the second and third intracellular loops, as well as the C terminal tail of GPCRs have been studied using point mutations and chimeric receptors (368). While several studies report a loss of activation in mutated receptors, others report the production of constitutively active receptors. This has led some investigators to the interpretation that the overall conformation of these regions may be more important for G protein activation than specific amino acids. Although the regions of C5L2 required for G protein coupling and activation are not yet known, an investigation of the C terminal tail has been carried out for C3aR (371). This study showed that a Ser449 to Ala mutation led to almost complete loss of coupling to both G_{16} and G_o. While other Ser/Thr residues in this region appear to be targets for GRK phosphorylation (372) and internalization (371), Ser449 may either be directly involved in G protein

coupling, or important in maintaining a conformation that allows coupling. Interestingly, this Ser is conserved in C5L2, based on the alignment (Figure 6.1), and therefore provides a target for investigation of C5L2-G protein interaction.

Another intracellular region shown to be important in G protein activation, is an Asp-Arg-Tyr (DRY) motif at the boundary of TM3 and the second intracellular loop. This motif is highly conserved in Class A GPCRs, particularly the aminergic GPCRs (368). While the aspartic acid is thought to stabilize the inactive state of GPCRs, the more highly conserved arginine is critical for G protein activation in many GPCRs (373). The fact that C5L2 has a Asp-Leu-Cys (DLC) sequence (Figure 6.1), rather than the conserved DRY motif, has led Okinaga *et al.* to conclude that C5L2 is obligately uncoupled from G proteins and must, therefore, be a nonsignaling receptor (353). Besides the fact that our data clearly show C5L2 function, and suggest G protein signaling, there are some reported exceptions to the DRY motif paradigm. Mutation of the arginine in the α2A (373) and β2 (374) adrenergic receptors show that the arginine is not required for G protein activation by these GPCRs. In the CB2 cannabinoid receptor, mutation of the homologous arginine only partially reduced activity (375), while arginine mutation in the type 1A angiotensin II receptor abolished coupling to some G proteins but not others (376).

Interestingly, the Class B GPCRs have a YL motif predicted to be in the same position as the first two amino acids of the Class A DRY motif. Mutation of the leucine in the vasoactive intestinal peptide 1 (VPAC1) receptor led to a pronounced impairment of G protein activation (377). Although highly

speculative, perhaps the existence of a leucine in both C5L2 and the VPAC1 receptor indicates some rare, remaining similarity after the evolutionary divergence of the GPCR classes. Determining the role of the DLC sequence of C5L2 is another area that may yield understanding of the mechanism of C5L2 function.

Phosphorylation and Internalization of GPCRs

Ligand binding to a GPCR leads not only to G protein activation, but also to rapid desensitization (350,352). Phosphorylation by second messenger kinases, such as PKC, of serine and threonine residues in the cytoplasmic loops and C-terminal tail impairs receptor-G protein coupling, and thus provides a feedback mechanism to limit the signaling of a receptor (352). Phosphorylation by second messenger kinases is not dependent on ligand activation of the GPCR, therefore receptors for other ligands can be desensitized by this heterologous process (352). In contrast, phosphorylation by GRKs only occurs on ligand-activated receptors (350,352), and may not be sufficient for desensitization (378). Rather, GRK phosphorylation promotes binding of β-arrestin (βarr), which sterically blocks G protein coupling to the receptor (378). βarr also interacts with clathrin and other proteins involved in endocytosis, to facilitate endocytosis of the receptor in clathrin coated pits (350,379).

Mutation of ten Ser/Thr residues in the C-terminal tail of C3aR indicated that four of them, Ser465/470 and Thr463/466, were important in receptor internalization, while the others were not (371). While the positions of several

Ser/Thr in C5L2 do not match exactly those in C3aR (Figure 6.1), they may be involved in βarr binding and receptor internalization.

The binding of βarr to GPCRs may be either stable or transient, depending on the βarr isoform involved. GPCRs that bind βarr1 and βarr2 equally well, such as the angiotensin and vasopressin receptors, internalize in a stable complex with βarr and are inefficiently recycled back to the cell surface (352,378). A stable GPCR-βarr complex retards dephosphorylation, and thus may favor degradation of the receptor (352). GPCRs that preferentially bind βarr2, such as the β2 adrenergic and dopamine receptors, dissociate rapidly from βarr upon internalization, and are recycled rapidly to the plasma membrane. The Ser/Thr clusters in the C-terminal tail of GPCRs determine the stability of the interaction with βarr.

The photomicrographs of fluorescently tagged βarr in Figure 5.3 were taken at several time points after addition of ASP. While the punctate pattern at the plasma membrane of the C5L2-expressing cells is clear at 2.5 and 5 minutes, the fluorescent spots are largely internal at 15 minutes, indicating that C5L2 has been internalized by this time. At 20 and 40 minutes, βarr is associated with larger vesicles, which may be lysosomes. This suggests a stable interaction with C5L2, and therefore possibly degradation of the receptor rather than recycling to the plasma membrane. Experiments with dyes that fluoresce in the acidic environment of the lysosome should determine whether or not these larger vesicles are indeed lysosomes.

Besides its role in internalization and recycling of receptors, βarr also acts as a scaffold for signaling proteins such as Src tyrosine kinases and members of the MAPK family, such as ERK1/2 (352,379). ERK1/2 activation of other kinases leads to transcriptional activity, thus scaffolding and activation of this pathway by βarr may be the mechanism by which ASP stimulates differentiation of adipocytes. βarr scaffold complexes appear to be relatively stable (352), suggesting that C5L2 may recycle to the cell surface relatively slowly, if at all.

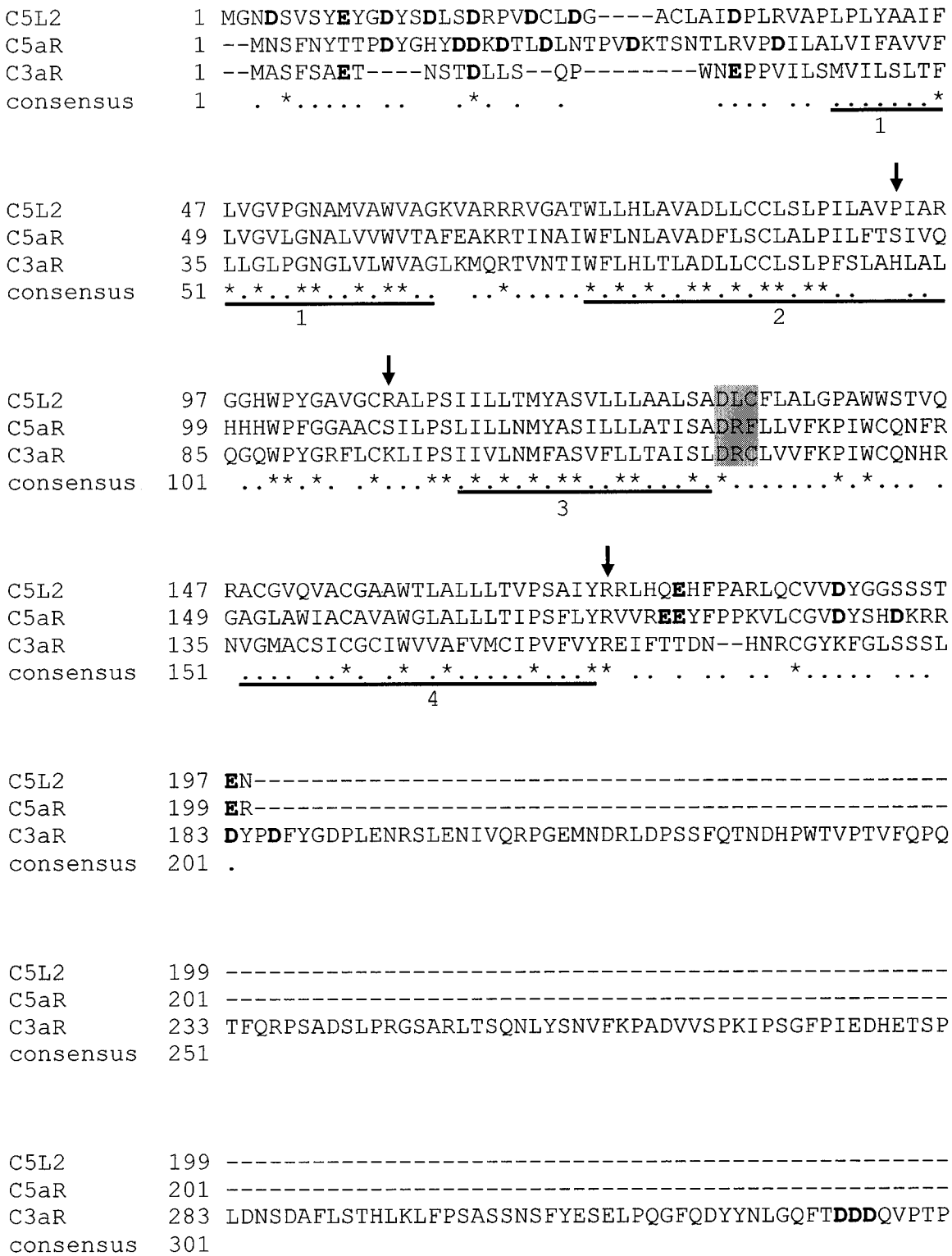
Conclusion

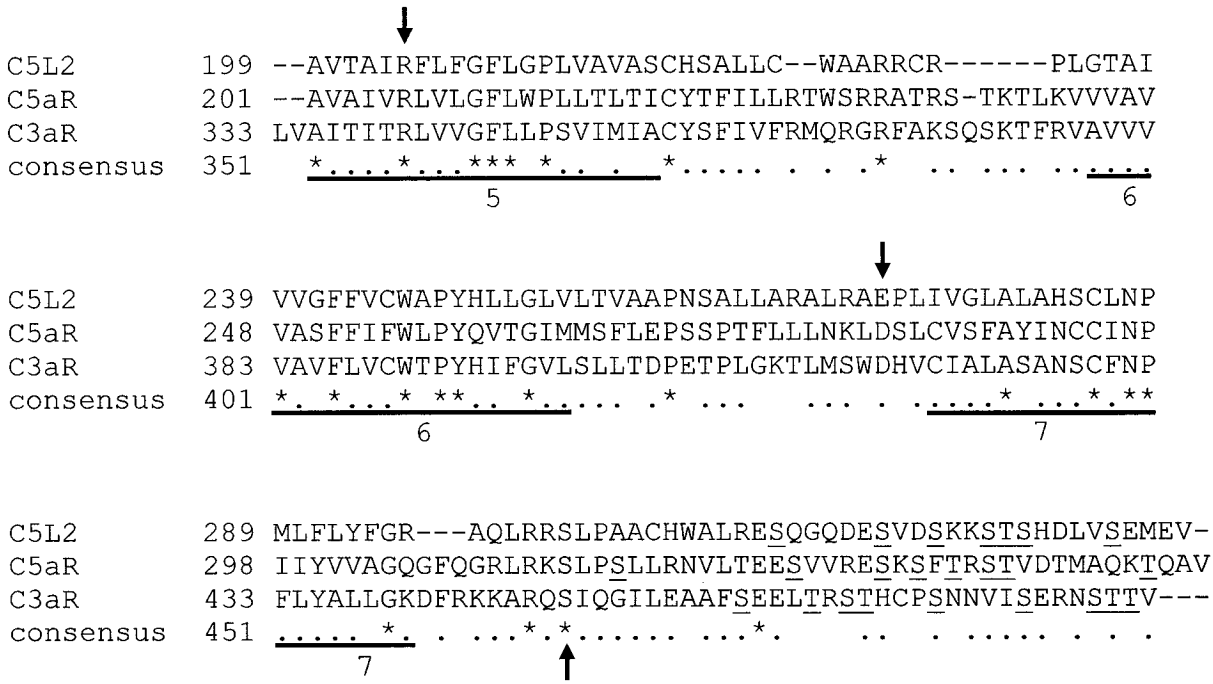
While there is much left to learn about the physiological role of ASP in humans, the information gained to date suggest that ASP is not only an important regulator of energy storage and adipose tissue metabolism, but also may play a role in obesity and related diseases. The identification of the ASP receptor is a critical step in understanding the mechanisms of ASP action. Further investigation of C5L2 activation and signaling, particularly ligand binding, may aid in finding C5L2 antagonists and exogenous agonists which could be used to modulate the ASP pathway. Such agents may eventually be useful as pharmacological aids for the treament of obesity or dyslipidemias.

The knowledge gained from the studies of related receptors, while not necessarily applicable to C5L2, nevertheless provides a good starting point for studying C5L2. Other areas of study related to ASP function are already underway. These include the ASP intracellular signaling cascade (discussed only briefly above), hormonal regulation of C5L2 expression, a search for natural

mutations of C5L2 in human subjects with hyperapoB, and further studies in knockout mice (possibly including a C5L2 knockout). Together these studies will place the ASP pathway, including C5L2, in its proper physiological context.

Figure 6.1 Alignment of C5L2, C5aR and C3aR Protein Sequences





KEY

Bold	Putative extracellular ligand binding sites
↓	Putative transmembrane ligand binding sites
Underlined	Putative C-terminal phosphorylation sites
X	"conserved" DRY motif
↑	Putative G protein interacting site
—	Transmembrane domains

Based on the number of amino acids in C5L2:
C3aR has 47% homology
C5aR has 53% homology

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APPENDIX

Radioisotope Permit

Biohazard Permit

Centre universitaire de santé McGill
McGill University Health Centre

Internal Permit #: 5-0012-05

Permit Holder: Katherine Cianflone **Office:** H7.30

Department: Cardiology Research **Telephone:** 35426

(A) Location

Room	Classification
H7.25	Radioactive Materials Area
H7.31	Basic
H7.33	Radioactive materials Area
H7.41	Basic
H7.88A	High
H7.36	Storage

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
C14	22.2	185
H3	22.2	185
I125	74	74

(C) Authorised Users

Last Name	First Name	P32	P33	S35	H3	C14	Fe59	I125	Ca45	Co57	Cr51	Na22	Rb86
Cianflone	Katherine	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
Cui	Wei	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
Faraj	May	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
Kalant	David	☐	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐
Legakis	Helen	☐	☐	☐	☑	☑	☐	☐	☐	☐	☐	☐	☐
Maslowska	Magdalena	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
McLaren	Robin	☐	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐
Paglialinga	Sabina	☐	☐	☐	☑	☑	☐	☐	☐	☐	☐	☐	☐
Smith	Jessica	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Vu	Hai	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
Zakarian	Robert	☐	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐

(D) Conditions

Conditions
Only R. Zakarian and D. Kalant are authorised for manipulation of > 135 uCi of I125

The internal permit holder and the persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the laboratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in conformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of our CNSC licence is posted at RVH Research secretariat office and is also available at Radiation Protection Service (room T9.100, local 43866).

Approved by:

Date issued: Monday, February 02, 2004

Expiration date: February 28, 2006

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Katherine Cianflone TELEPHONE:

   ADDRESS: FAX NUMBER

   E-MAIL:

   DEPARTMENT: **Medicine (Cardiology Research)**

   PROJECT TITLE: **Hormonal regulation and structure: Function of C5L2 receptor in adipose tissue.**

2. FUNDING SOURCE: CIHR ☒☒☒ NSERC ☐ NIH ☐ FQRNT ☐ FRSQ ☐

   INTERNAL ☐ OTHER ☐ (specify) _____

   Grant No.: **Accepted – waiting for number** Beginning date **July 2003** End date **June 2006**

3. Indicate if this is

   ☒☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
   Approval End Date _____

   New funding source: project previously reviewed and approved under an application to another agency.

   Agency _____ Approval End Date _____

   New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".
Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: date: 06 July 2003
(day month year)

COIMBATORE B. SRIKA
Chairperson, Biohazards Committee: _____ (SIGNATURE) date: _____
(day month year)

Approved period: 1 year beginning 01 (day) 07 (month) 03 (year) ending 30 (day) 06 (month) 04 (year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
David Kalant	Medicine (RVH)		X			
Magdalena Maslowska	Medicine (RVH)		X			
Hai Vu	Medicine (RVH)		X			
Robin MacLaren	Experimental Medicine				X	

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Katherine Cianflone Phone No: work: X35426 home: (540) 357-1904

Name: David Kalant Phone No: work: X 35425 home: (514) 693-9423

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group:

- Bacteria: E. Coli strains Top 10 F', XL-10 Gold, GT116, electro-10 Blue - Level 1
- Viral Vectores: MMLV based vectors for transduction of mammalian cells - Level 2
- Yeast: Pichia pastoris expression system - Level 1
- Mammalian cells/tissue: 3T3-L1, 3T3-F442, HEK293/17, SW872, human skin fibroblasts, human adipose tissue, tissues extracted from mice - Level 2

ii) the procedures involving biohazards - General precautions for all:

- Use disposable latex gloves, masks for viral work, bleach in liquid traps, disposal of materials into biohazard boxes, or after treatment with bleach or autoclaving (viral vectors).
- Use of appropriate biocontainment (mammalian cells, viruses) or laminar (bacteria, yeast) hoods. Working areas wiped down with Roccal disinfectant (cells) or ethanol (bacteria). Use of incubator specific for cells or bacteria

iii) the protocol for decontaminating spills: All spills are immediately wiped up with absorbent paper and disposed in biohazard box. Area wiped down with Roccal or Ethanol.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

YES

9. What precautions are being taken to reduce production of infectious droplets and aerosols?

Minimise the time that flasks are open. Cap all containers when mixing. Work in appropriate hoods.

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
RVH	H7 - 29	Canadian Cabinets Company	BM4-2A-49	11185	Aug. 2, 2003