Secretion in 3T3-L1 adipocytes

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BY

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Abstract

Adipocytes secrete a range of proteins, called adipokines, with important roles in a variety of biological processes such as metabolism, cardiovascular function and immunity. ACRP30 (also known as adiponectin) is an adipokine that displays anti-diabetic and anti-inflammatory properties and may prevent atherosclerosis. It is secreted exclusively by adipocytes and circulating levels of ACRP30 are reduced in obesity and diabetes. Although this protein has been widely studied, little is known about the mechanisms of secretion of ACRP30 in adipocytes.

Here I have examined secretion of ACRP30 in 3T3-L1 adipocytes with the aim of understanding the secretory pathways used by ACRP30 as it traffics to the cell surface. Using intracellular localisation techniques I have shown that ACRP30 overlaps with transferrin-receptor positive membranes, evidence that ACRP30 traffics via the transferrin-receptor positive endosomal system. This was supported by results showing that ablation of the transferrin-receptor positive endosomes strongly inhibited ACRP30 secretion. I have also investigated the effects of overexpressing mutant forms of the small GTPases Rab11 and Arf6. Overexpression of Rab11 S25N significantly reduced basal and insulin-stimulated ACRP30 secretion and I have demonstrated that Arf6 plays a role in ACRP30 secretion.

With the aim of identifying new adipokines, I have conducted a proteomic analysis of the proteins secreted by 3T3-L1 adipocytes. In this study, I identified 25 proteins that had not previously been identified as adipocyte-secreted. In functional studies, two of these proteins, orosomucoid and Nm23 could be shown to affect insulin-stimulated glucose uptake. In monocyte adhesion assays orosomucoid had anti-inflammatory effects.

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Author's declaration

I declare that the work described in this thesis is entirely my own, unless otherwise acknowledged. The thesis is of my own composition and has not, wholly or in part, been submitted for any other degree.

Mairi Clarke

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Abbreviations

ARF	ADP-ribosylation factor
APPL1	Adaptor protein containing PH domain, PTB domainand Leucine zipper motif 1
C/EBP	CCAAT/enhancer binding protein
DeGlc	2-deoxy-D-glucose
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FCS	Fetal calf serum
GDI	GDP dissociation factor
GEF	Guanine nucleotide exchange factor
GLUT	Glucose transporter
GSK3	Glycogen synthase kinase 3
GTP	Guanosine 5'-triphosphate
HDM	High density microsome
HEPES	*N*-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
h	Hours
HRP	Horseradish peroxidase
HUVEC	Human vascular endothelial cells
IgG	Immunoglobulin gamma

IRS	Insulin receptor substrate
IL	Interleukin
KRP	Kreb's ringer phosphate buffer
LDL	Low density lipoprotein
LDM	Low density microsomes
min	Minutes
mA	Milliamps
NSF	*N*-ethylmaleimide-sensitive fusion protein
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PLD	Phospholipase D
PBS	Phosphate buffered saline
PI 3-kinase	Phosphatidylinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-triphosphate
PM	Plasma Membrane
PPARγ	Peroxisome proliferator-activated receptor γ
RBP4	Retinol binding protein 4
SDS	Sodium dodecyl sulphate
SNAP	Soluble NSF attachment protein
SREBP	Sterol regulatory element binding protein
SRP	Signal recognition peptide

TCA	Trichloroacetic acid
TfR	Transferrin receptor
TGN	Trans-Golgi network
TNF	Tumour Necrosis Factor
v/v	volume/volume
w/v	weight/volume

Chapter1 General Introduction

1.1 Adipose tissue and Adipokines

Adipose tissue is found in mammals and has long been known as the main energy reserve in the body. Energy is stored in the form of triglycerides in adipocytes, ready to be used when caloric expenditure exceeds intake. Adipose tissue is composed of several different cell types. As well as adipocytes, adipose tissue contains adipose tissue matrix and a stromal vascular fraction (SVF) that comprises pre-adipocytes, endothelial cells and macrophages. Adipose tissue takes the form of loose connective tissue, and is found in mammals, fish, birds, reptiles and amphibians. In mammals, fat is found in distinct locations in the body and serves different functions in these different locations. Some depots of fat have a structural or protective role, like the fat pads found in hands, feet, joints and around the eyes. Subcutaneous fat is the layer of fat found just underneath the skin. Visceral fat is a depot found surrounding the internal organs and is the main fat depot associated with the development of type2 diabetes and cardiovascular disease (Kissebah and Krakower, 1994).

Energy is stored in adipocytes in the form of triglycerides, which are broken down to fatty acids and glycerol by lipases (lipolysis) (Coppack et al., 1994). Most of this stored energy comes from ingested triglycerides, but a small amount of fatty acids are synthesised in the liver and adipocytes. When other cells in the body require energy, triglycerides stored in adipocytes are broken down by the action of lipases within adipocytes. Fatty acids can then be released and used as a fuel by other cells. Fatty acids circulate as free fatty acids (FFA), bound to albumin and are taken up, by specific transporter proteins in muscle and liver, and oxidised, to provide ATP, or taken up again by adipocytes for storage as triglycerides (Large et al., 1998).

Recently it has been recognised that as well as their role in energy storage, adipocytes regulate many diverse processes through their secretion of a range of endocrine and paracrine signalling molecules called adipokines. These adipokines have a role in regulating energy metabolism, insulin sensitivity in the liver and skeletal muscle (Abel et al., 2001), and many other biological processes such as angiogenesis, blood-presssure, bone formation, inflammation and the acute-phase response. Whilst the term 'adipokines' has been used to describe proteins secreted by adipose tissue, it is generally accepted that it refers to those proteins secreted by adipocytes (Trayhurn and Wood, 2004). Since the

discovery that adipocytes were a site of secretion of sex-hormones in 1987 (Siiteri, 1987), many more adipokines have been identified, notably adipsin, leptin and TNF-α.

Adipsin was found to be secreted by murine adipocytes but down regulated in some types of obesity (Flier et al., 1989). Leptin was identified as an important regulator of metabolism (Friedman and Halaas, 1998) and TNF-α was the first inflammatory cytokine found to be secreted by adipocytes (Hotamisligil et al., 1995). Several adipokines are pro-inflammatory cytokines such as TNF-α, IL-6, angiotensinogen which promote endothelial dysfunction, through increases in endothelial adhesion, platelet activation and eventual plaque formation, leading ultimately to cardiovascular disease (Lau et al., 2005) and others like adiponectin, resistin and RBP 4 (Yang et al., 2005) have been identified as adipokines implicated in contributing to the development of insulin resistance. Adipocytes also secrete acute-phase proteins like C reactive protein (Calabro et al., 2005) and the complement factors adipsin (Cook et al., 1987) and ASP (Maslowska et al., 1997) .

Because these fat-secreted proteins have metabolic effects on other cells and tissues, they have the potential to explain the links between obesity and metabolic diseases, there is a great deal of interest in their biology and physiological actions. Below is a summary of what is known of the biology and actions of the better-studied adipokines, and the role they might play in obesity and its associated diseases.

1.1.1 Adiponectin

Adiponectin, also called ACRP30, was first described as 30 kDa protein secreted exclusively by adipocytes whose mRNA was induced 100-fold during adipocyte differentiation (Scherer et al., 1995). It is thought to be the most abundantly secreted adipokine and circulates in normal serum at a concentration of between 5 and 30μg/mL. Unlike many other adipokines, serum concentration decreases with the progression of obesity, cardiovascular disease and insulin resistance (Arita et al., 1999; Hotta et al., 2000). Treatment of insulin resistant humans and mice with the insulin sensitising drug thiazolidinedione (TZD), results in increased circulating adiponectin levels (Combs et al., 2002)

Adiponectin consists of an amino terminal signal sequence, a collagenous domain and a C-terminal globular domain (Shapiro and Scherer, 1998). The C-terminal globular head

domain of adiponectin shares structural homology with complement C1q and the globular domain of several collagens. The crystal structure of adiponectin shares structural homology with TNF-α, although they share no sequence homology. In its most basic form, adiponectin is found as a trimer of three 30kDa monomers. These trimers multimerise further, through disulphide bonds in the collagenous region, to form higher molecular weight structures (Figure 1.1). The protein circulates in serum as a hexamer of low molecular weight and a larger high molecular weight structure of 12-18 subunits (Pajvani et al., 2003). Findings from structure-function studies show that the trimeric form of the protein possesses greater bioactivity, suggesting that the higher molecular weight forms may be precursors awaiting proteolytic cleavage to the active trimeric form. Berg *et al* (Berg et al., 2001; Pajvani et al., 2003) demonstrated that full length recombinant adiponectin decreased serum glucose by decreasing hepatic glucose output when administered to normal and obese/diabetic mice. This action on the liver is dependent on the presence of the collagenous domain and is therefore, only seen with the full-length protein. Administration of the proteolytically cleaved globular head increases fatty acid oxidation and glucose uptake in muscle cells *in vitro* (Fruebis et al., 2001), an effect which is mediated by the activation of AMPK (Yamauchi et al., 2002). There is evidence that adiponectin is necessary for the anti-diabetic actions of the PPAR-binding drugs called TZDs (Thiazolidinediones). TZDs have been shown to raise serum levels of adiponectin and increase insulin sensitivity. Adiponectin has also been shown to have potential antiatherogenic properties, binding to collagen in the subendothelium of damaged vascular tissue (Okamoto et al., 2000) and suppressing monocyte adhesion to endothelial cells by inhibiting the expression of the adhesion molecules intracellular adhesion molecule-1, vascular cellular adhesion molecule-1 and E-selectin. This action of adiponectin may be via suppression of NF-κB, since adiponectin has been shown to inhibit activation of NF-κB by the inflammatory mediator TNF-α, by inhibiting phosphorlation of I-κB-α (Ouchi et al., 2000).

There is evidence that adiponectin is necessary for the anti-diabetic actions of the drugs called TZDs (Thiazolidinediones). TZDs have been shown to raise serum levels of adiponectin

Adiponectin acts by binding to one of two receptors AdipoR1, which is expressed in skeletal muscle and AdipoR2, which is predominantly expressed in liver (Yamauchi et al., 2003). Adiponectin binding to the receptor leads to the interaction of the adaptor protein APPL1 (Mao et al., 2006). AdipoR1 and AdipoR2 are seven transmembrane domain

proteins and unlike G protein-coupled receptors their N-terminus is internal and the C-terminus is external. Expression of Adipor1 or Adipor2 in liver can reverse insulin resistance and diabetes in db/db (leptin receptor null) mice. Expression studies have also shown that AdipoR1 is closely associated to activation of AMPK pathways, regulating the inhibition of gluconeogenesis, whilst activation of AdipoR2 is more associated with activation of PPAR-α pathways, and thus inhibition of inlammation and oxidative stress (Yamauchi et al., 2007). The plant homologue of the adiponectin receptor, PHO36, is also a seven transmembrane domain protein and binds a membrane protein osmotin, that regulates phosphate and lipid metabolism (Narasimhan et al., 2005), although the two proteins share no homlogy.

The glycosylphosphatidylinositol-anchored protein T-Cadherin has also been identified as a receptor for hexameric and high molecular weight forms of adiponectin (Hug et al., 2004).

There is an aparent link between Adiponectin levels and insulin resistance. Plasma levels od adiponectin are reduced in obese db/db mice (leptin receptor- deficient mose model for obesity) (Hu et al., 1996) and decreased in patients with cardiovascular disease (Hotta et al., 2000), and the metabolic syndrome (Trujillo and Scherer, 2005), all states which are associated with insulin resistance. It is not known whether this link is causitive, although the insulin sensitising effects of adiponectin have been demonstrated by several groups. Fruebis et al have shown that the globular 30kDa adiponectin, when administered to mice on a high fat diet, resulted in weight loss and a decrease in plasma free fatty acids (Fruebis et al., 2001). Yamauchi et al showed that by administering physiological doses of adiponectin to lipoatrphic/diabetic mice they could ameliorate the state of insulin resistance (Yamauchi et al., 2001). Berg et al shsowed that administration of adiponectin to both wild type and ob/ob (a mouse model for Type 2 diabetes) increased hepatic insulin action and a suppression of glucose production (Berg et al., 2001). These three independent studies all provide evidence that a decrease in adiponectin in the obese/diabetic state may be causal in the development of insulin resistance.

Reduced levels of circulating adiponectin have been observed in obesity, insulin resistance and type 2 diabetes amongst human populations (Hotta et al., 2000; Trujillo and Scherer, 2005) and also in cardiovascular disease (Kojima et al., 2003; Kumada et al., 2003). A recent study of the relationship between adiponectin levels and cardiovascular disease, based on data from the Strong Heart Study, the largest study of cardiovascular disease in American Indians. This group are a high-risk group for obesity, insulin resistance, type 2

diabetes and cardiovascular disease. Increased serum levels of adiponectin were not found to predict cardiovascular disease in this group (Lindsay et al., 2005). Differences in results form these various studies may be indicative of differences in the populations studied.

1.1.2 Adipsin

Adipsin is a serine protease that is secreted by adipocytes and macrophages and whose serum levels are reduced in mouse models of obesity (Cook et al., 1987). Also known as complement factor D. Adipsin is constitutively secreted (Millar et al., 2000) and is a component of the alternative complement pathway, cleaving C3-bound factorB (White et al., 1992), therefore Adipsin/FactorD is a protein of the immune system, that is also highly expressed in adipose tissue.

1.1.3 Acylation stimulating protein

ASP or C3a-des Arg ASP is generated through the interaction of precursor C3, cofactor B, and the serine protease enzyme, adipsin, all three of which are secreted by adipocytes. ASP stimulates triglyceide synthesis in adipocytes and its circulating levels are increased in obesity, diabetes (Koistinen et al., 2001) and coronary heart disease (Cianflone et al., 1997).

1.1.4 Resistin

Resistin is the product of the *RETN* gene and belongs to a family of cysteine-rich secreted proteins called the RELM family. Mouse resistin is a 114 amino acid polypeptide with a hydrophobic 20 amino acid N-terminal signal sequence that is cleaved before secretion. Mouse resistin circulates as a homodimer of these 94 amino acid peptides linked by a disulphide bond.

Resistin was first identified as a protein secreted by murine adipocytes and circulating in mouse serum and whose serum levels are increased in obesity (Steppan et al., 2001b). Secretion of resistin in 3T3-L1 adipocytes is up regulated by the induction of C/EBP-α but reduced by the induction of PPAR-γ. This regulation of secretion is controlled via the PI3-kinase and MAP kinase pathways (Song et al., 2002). Secretion of resistin in stimulated by insulin (McTernan et al., 2003) and down regulated by TNF-α (Fasshauer et al., 2001). Called resistin (for resistance to insulin) and identical to the protein FIZZ3, it was found to be up regulated during adipocyte differentiation and down regulated in mature adipocytes

treated with the anti-diabetic drug TZD and was thus identified as a potential link between obesity and diabetes (Steppan et al., 2001b). Increased levels of circulating resistin were observed in obese mice. Treatment of these mice with a PPAR-γ agonist reduced circulating resistin levels and injection of antibodies against resistin improved the insulin resistant state of the animals. Human fat cells do not produce resistin (Nagaev and Smith, 2001), and although it is released by adipose tissue, probably by macrophages, is not thought to influence insulin sensitivity (Utzschneider et al., 2005). There is evidence that resistin acts as a pro-inflammatory cytokine, up regulating IL-6 and TNF-α in arthritis (Bokarewa et al., 2005).

1.1.5 Leptin

Leptin is the protein product of the *ob* gene (Zhang et al., 1994). It plays a role in regulating energy intake and expenditure by controlling appetite and metabolism and as a signal of sufficiency of energy (Pittas et al., 2004). It is mainly produced in adipose tissue and is found circulating in plasma in levels that correlate with percentage body fat (Considine et al., 1996). Circualtin levels of Leptin decline with weight loss, where reduced leptin levels are associated with increased appetite and decreased energy expenditure. A state of leptin resistance is observed in many cases of obesity, where high serum levels of leptin are ineffective in reducing appetite (Flier, 1998). Leptin resistance in obesity is thought to be mediated by SOCS3, a member of the SOCs family of proteins (suppressors of cytokine signalling) (Howard and Flier, 2006).

There are two widely studied mouse models that are homozygous for single-gene mutation. *ob/ob* mice are deficient for leptin protein and *db/db* mice are deficient for the leptin receptor. Both mouse models display obesity, insulin resistance, hyperglycaemia and hyperlipidemia. Intravenous injection of leptin to o*b/ob* mice causes weight loss and reduces food intake and serum levels of glucose and insulin (Pelleymounter et al., 1995).

Leptin secretion from adipocytes is increased by treatment with TNF-α and insulin although it is not known whether the insulin-dependent increase is caused by an increase in the rate of constitutive secretion, or if Leptin is stored inside the cell and transported to the cell surface in response to the insulin stimulus (Mora and Pessin, 2002). Leptin circulates bound to a soluble form of its receptor. This binds to a transmembrane receptor Ob-R, found in various tissues including adipocytes, liver and skeletal muscle and in the central nervous system and are concentrated in an area of the hypothalamus called the arcuate nucleus that is known to be involved in regulating food intake (Tartaglia et al., 1995). In

skeletal muscle leptin acts by phosphorylating AMPK, both directly and through the central nervous system. Leptin also inhibits the activity of acetyl CoA carboxylase (ACC), thus stimulating fatty-acid oxidation in muscle (Minokoshi et al., 2002). Cohen et al showed that leptin's action in liver depends on down-regulation of stearoyl-CoA desaturase-1 (SCD-1), an enzyme that catalyses the biosynthesis of fatty acids (Cohen et al., 2002).

Leptin is also an important hormone, regulating various processes including immune function, hematopoiesis, bone development and angiogenesis (Margetic et al., 2002). It plays a role in immune function, by affecting cytokine production, it affects endothelial cell growth and angiogenesis, accelerates wound healing and plays an important role in bone development. Leptin deficient ob/ob mice also display increased bone mass. This effect probably results from leptin's influence on the sympathetic nervous system (Cock and Auwerx, 2003).

1.1.6 Visfatin

Visfatin is a protein secreted by visceral fat, whose expression increases with the development of obesity. It is the same as pre-B-cell colony enhancing factor, a 52kD protein secreted by lymphocytes. Visfatin mimics the effects of insulin in cultured cells and lowers plasma glucose levels in mice, and has been shown to bind to insulin receptors (Fukuhara et al., 2005).

1.1.7 TNF-α

TNF-α is a pro-inflammatory cytokine that was first identified as a secreted factor that responsible for the necrosis of tumours (Carswell et al., 1975). It is expressed as a 26kDa transmembrane precursor, which is cleaved to release the 17kDa polypeptide. Three of these monomers associate to form a homotrimer shown in Figure 1.1. TNF-α binds to two cell surface receptors TNFR1 and TNFR2 (Vandenabeele et al., 1995), and recruits several adaptor proteins that can induce signalling pathways leading either to apoptosis or to the activation of the NFκβ and JNK transcription factors. Activation of these factors induces cellular responses such as inflammation, differentiation and proliferation (Dempsey et al., 2003). During activation of the inflammatory response endothelial cells are an important target for the actions of TNF-α, including the induction of adhesion of leukocyte adhesion molecules (Macconi et al., 1995) and the modulation of nitric oxide production (Yoshizumi et al., 1993). TNF-α is secreted by macrophages, lymphocytes and adipocytes. In rodent

models of obesity there is evidence to suggest a positive correlation between circulating TNF-α levels and obesity or insulin resistance (Hotamisligil et al., 1995), (Moller, 2000). Adipocytes express both TNFR1 and TNFR2 and adipocyte derived TNF-α is thought to act mainly in an autocrine manner, increasing lipolysis and releasing FFAs into the circulation (Hotamisligil et al., 1995), thus contributing to insulin resistance.

1.1.8 Retinol-binding protein 4

Retinol-binding protein 4 (RBP4) is an adipocyte-secreted member of the lipocalin family and is associated with obesity and diabetes. Serum levels of RBP4 are increased with insulin resistance. Adipose-specific Glut4 knockout mice show insulin resistance, not only in adipose, but also in muscle and liver. These mice also show elevation of adipose-secreted RBP4. Overexpression of RBP4 increases insulin resistance in liver and muscle, and treatment with an insulin-sentising drug, also normalised RBP4 levels. Increased serum RBP4 induces expression of the gluconeogenic liver enzyme phosphoenolpyruvate carboxykinase (PEPCK), which reduces insulin signalling and lowers glucose uptake by the liver, thus contributing to the disease-state of type 2 diabetes (Graham et al., 2006). The above experiments suggest that RBP4 is an important link between obesity and insulin-resistance, being secreted by fat tissue and having a regulatory effect on insulin sensitivity in other tissues. RBP4 is also a human adipokine, but its involvement in the link between obesity and disease has yet to be confirmed in humans (Janke et al., 2006). This recently discovered adipokine appears to be one of the sought after links between obesity and insulin resistance.

1.1.9 C-reactive protein

C-reactive protein (CRP), an acute-phase reactant, is considered one of the best predictors of future cardiovascular disease. CRP comprises 5 23kDa subunits and is produced mainly in the liver in response to inflammatory cytokines (Hirschfield and Pepys, 2003), but is also produced by respiratory-tract epithelial cells (Gould and Weiser, 2001), coronary artery smooth muscle cells and when stimulated by certain cytokines, adipocytes. CRP has been found to co-localise with complement components in atherosclerotic plaques (Calabro et al., 2005), and can activate the expression of cell-surface adhesion molecules in endothelial cells.

1.1.10 Significance of Adipokines

It is clear that fat tissue is more complex than first thought and that it has a role to play in the development of several metabolic diseases. The adipokines listed above are all secreted by adipose tissue and play an important role in regulating cell growth and metabolism and altered levels of secretion of these adipokines is often seen in insulin resistance and obesity. Circulating levels of the adipokines ACRP30 and leptin are thought to be crucial regulators in the development of insulin resistance and the metabolic syndrome by controlling fatty acid oxidation in muscle (Minokoshi et al., 2002; Yamauchi et al., 2002).

The regulation of adipokine synthesis and secretion is complex. Unlike the regulated secretion seen in neuroendocrine cells, there appears to be a lack of a clear stimulus-triggered secretory pathway in adipocytes, and there is still much to be understood about the control of secretion of adipokines.

Many new adipokines have been discovered recently and their importance in disease investigated, and some, like RBP4 have revealed a possible link between obesity and insulin resistance. It is likely that there remain yet undiscovered adipokines with a role to play in the link between obesity, insulin resistance, diabetes and heart disease. Therefore, an important area of research will be the discovery of new adipokines and investigation of their contribution to insulin resistance, diabetes and cardiovascular disease.

1.2 Obesity, Insulin resistance and the metabolic syndrome

1.2.1 Epidemiology of Obesity

Figures published by the World Health Organisation estimate that in the year 2005 there were in the region of 1.6 billion overweight adults worldwide (with a BMI > 25kg/m^2), and 400 million clinically obese adults (with a BMI > 30kg/ m^2). There is a prediction that one in three children born this century will develop diabetes in their lifetime. In Europe Obesity is considered one of the greatest public health challenges facing society, being responsible for 2-8% of total health costs and in the region of 10% of deaths. Perhaps the

most worrying trend associated with the obesity epidemic is the rate of increase of the disease in the developing world. Obesity was previously considered to be a problem of the more affluent 'Western' cultures, but is now increasing most rapidly in poorer countries where governments can ill afford the extra financial burden that comes with its associated health problems. Obesity leads to many serious health complications and the risk to health increases with BMI. The main chronic conditions for which obesity is a risk factor are: cardiovascular diseases (CVD) which kills 17 million people per year worldwide; diabetes, whose incidence is predicted to increase worldwide by 50% in the next 10 years; musculoskeletal disorders and some cancers (particularly breast and gastro-intestinal) (data obtained from The World Health Organisation (http://www.who.int). There is also a worrying increase in the rates of obesity in children and young people. It is predicted that by 2010, 1 in 10 European children will be obese, causing an increase in the chronic illnesses mentioned above, diseases previously thought only to apply to adults. Obesity will also reduce life expectancy in these young people, with a projected average of 5 years lower life expectancy for men by 2050 (data taken from WHO press release EURO/11/06).

1.2.2 Insulin resistance and metabolic syndrome

Insulin resistance is described as a failure of target organs i.e. muscle, adipose tissue and liver to respond to the action of insulin, resulting in increased levels of blood glucose and increased insulin production by the pancreatic β-cells (Kahn, 1978). The accumulation of visceral fat is associated with the development of insulin resistance, type 2 diabetes and a cluster of disorders that pose an increased risk of cardiovascular disease, including hypercholesterolemia, hyperglycemia and hypertension. Taken together these disorders are known as the metabolic syndrome or insulin resistance syndrome (Reaven, 1988). These metabolic diseases could be caused by an excess of impaired fatty acids called non-esterified fatty acids (NEFA), which enter the circulation and are taken up by the liver, resulting in insulin-resistance in the liver. Visceral obesity often results in an excess of these NEFA, caused by atrophy of the intra-abdominal adipose tissue and large hyperlipolytic adipocytes that are resistant to insulin (Mittelman et al., 2002).

Circulating levels of the adipokines ACRP30 and leptin are thought to be crucial regulators in the development of insulin resistance and the metabolic syndrome by controlling fatty acid oxidation in muscle (Minokoshi et al., 2002; Yamauchi et al., 2002). In addition adipose tissue secretes a range of pro-inflammatory cytokines such as TNF-α, IL-6, PAI-1 and angiotensinogen, which promote endothelial dysfunction, through increases in

endothelial adhesion, platelet activation and eventual plaque formation, leading ultimately to cardiovascular disease (Lau et al., 2005). Adipocytes also secrete acute-phase proteins like C reactive protein (Calabro et al., 2005) and the complement factors adipsin (Cook et al., 1987) and ASP (Maslowska et al., 1997) .

1.3 Obesity and Inflammation

Obesity has been compared to a state of chronic low-grade inflammation, because of the increased serum levels of many pro-inflammatory cytokines (Yudkin et al., 1999). It has also been recognised, however, that obesity is accompanied by an infiltration into adipose tissue by macrophages and these are likely to contribute to the inflammatory state (Weisberg et al., 2003). Normal mature adipocytes and macrophages are derived from bone marrow stem cells: adipocytes from stromal or mesenchymal stem cells, and macrophages from haematopoietic stem cells. Hematopoietic stem cell give rise to blood cells (Abramson et al., 1977) and mesenchymal stem cells give rise to adipocytes, chondrocytes and osteocytes (Pittenger et al., 1999). Adipocytes and macrophages are distinct cell types, derived from distinct precursors and each with their own genetic and phenotypic characteristics. Despite their distinct characteristics and the fact that they are derived from different stem cells they do share a common developmental ancestry, as they have both developed from mesoderm (Prockop, 1997) and they also share many characteristics, including the secretion of pro-inflammatory cytokines like TNF-α ,IL-6 and the fatty acid binding protein aP2 (Boord et al., 2004). In certain conditions like obesity, the distinctions between them become less marked, as adipocytes lose a number of their defining characteristics and become more "macrophage-like". Macrophages express adipocyte proteins and vice-versa and the functions of these cells can, in some situations, overlap.

Obesity is marked by an increase in adipose tissue comprising adipocytes, preadipocytes and macrophages. The pro-inflammatory cytokines produced by macrophages and adipocytes have been implicated in the development of insulin resistance that often accompanies obesity. With an increase in adipocytes come an increase in cytokines, infiltration of macrophages, and an increase in levels of inflammatory cytokines.

1.4 Insulin signalling

Insulin is secreted by pancreatic β-cells in response to elevated blood glucose. It binds to tyrosine kinase receptors on the surface of adipose, liver and skeletal muscle cells. Binding of insulin to its receptor triggers the activation of various signalling cascades, resulting in a wide range of effects including gene expression, cell growth and differentiation, increased glucose uptake and glycogen synthesis in muscle and triglyceride storage in adipose tissue (Saltiel and Pessin, 2002). In the liver, insulin stimulates the storage of glucose as lipids and glycogen and inhibits the synthesis and release of glucose by regulating the expression of the enzymes that catalyse gluconeogenesis and glycolysis (Saltiel and Kahn, 2001).

The insulin receptor is a tetrameric protein comprising two α and two β subunits. The α-subunit of the insulin receptor contains the hormone binding region subunit and insulin binding activates the catalytic β-subunit of the receptor. This results in autophosphorylation of the β-subunits at several tyrosine residues and subsequent phosphorylation of other intracellular proteins, like the insulin receptor substrates (IRS 1 and 2), Shc and Cbl. Phosphorylation of these proteins results in the activation of various signalling pathways.

1.4.1 Insulin Signalling via PI 3-kinase

IRS provides docking sites for several SH2 containing proteins (White, 1997). Of particular importance is the lipid kinase, PI 3-kinase. Binding of the p85 regulatory subunit of PI3-kinase to the IRS brings it in proximity to the PM thus facilitating the phosphorylation of PtdIns (4,5)P_2 by the p110 catalytic subunit and resulting in the initiation of the PI 3-kinase cascade, which along with the MAP kinase pathway triggers downstream events that bring about the Insulin response (Figure 1.2). Phosphorylation of PtdIns (4,5)P_2 results in an increase in the intracellular level of the second messenger PtdIns (3,4,5)P_3 which then interacts with phosphoinositide-dependent kinase PDK1, a serine kinase that activates PKB/Akt. Akt is itself a serine/threonine kinase that phosphorylates a range of substrates, all of which play a part in the insulin response within the cell. GSK3 is inactivated by Akt phosphorylation resulting in increased glycogen sythesis. PKB/Akt and two atypical PKC isoforms ξ and λ are thought to be involved in translocation of the insulin dependent glucose transporter GLUT4 to the cell surface (Lizcano and Alessi, 2002). There is evidence however that activation of PI 3-kinase alone is not sufficient for the insulin-stimulated translocation of GLUT4 and the CAP/Cbl/TC10

pathway has been proposed as a possible PI 3-kinase independent form of insulin stimulated glucose transport (Jiang et al., 1998).

1.4.2 Insulin signalling via CAP/Cbl

In some cell types the proto-oncogene Cbl is phosphorylated in response to insulin binding to its receptor (Ribon and Saltiel, 1997). The Cbl protein, in a complex with CAP is then translocated to a lipid raft region of the PM. Here the phosphorylated CAP-Cbl complex recruits CrkII, along with the GEF C3G. C3G is then able to activate TC10, a Rho GTPase. It has been suggested that activated TC10 acts as a signal for PI 3-kinase independent translocation of GLUT4 vesicles to the PM (Baumann et al., 2000; Chiang et al., 2001). This role of the CAP/CBl/TC10 pathway as a PI 3-kinase independent pathway for insulin stimulated glucose transport has however been brought into question by experiments where CAP, Cbl and CrkII were depleted in 3T3-L1 adipocytes. Using siRNAs, under these conditions, insulin-stimulated glucose transport was not affected, suggesting that CAP, Cbl and CrkII are not involved in this process (Mitra et al., 2004).

1.5 Adipocytes

1.5.1 3T3-L1 Adipocytes

3T3-L1 adipocytes are derived from the 3T3-L1 preadipocyte cell line, selected from Swiss 3T3 mouse fibroblasts for its ability to differentiate into adipocytes. When growth arrested in the presence of foetal bovine serum they become rounded and accumulate cytoplasmic vesicles comprised of triglycerides (Green and Kehinde, 1975). This differentiation is enhanced when the cells are exposed to a cocktail of inducing agents resulting in the activation of the three transcription factors PPARγ, C/EBP, and ADD-1/SREBP-1 and their subsequent initiation of adipocyte specific protein expression (Spiegelman et al., 1993; Tontonoz et al., 1993; Rubin et al., 1978). When differentiated, 3T3-L1 adipocytes exhibit many of the biochemical and morphological properties of normal mouse adipocytes, and like mouse adipocytes, are insulin-responsive. 3T3-L1 adipocytes are a popular model used in the study of adipose biology. They are convenient source of reproducibly insulin-responsive adipocytes, and contain cells that represent only the pre-adipocyte and adipocyte fraction. Primary rodent adipocytes on the other hand, must be isolated from adipose tissue which contains several different cell types. The standard procedure for isolation of primary adipocytes from adipose tissue induces a significant inflammatory response, which results in changes in gene expression. Cultured

primary rodent adipocytes eventually exhibit down-regulation of insulin-responsive Glut4 and an increase in expression of Glut1(Gerrits et al., 1993).

1.5.2 Transcriptional regulation of adipocyte differentiation

PPAR-γ The adipocyte specific form PPAR-γ2 is a nuclear receptor that, when activated becomes a transcriptional activator. PPAR-γ is activated by binding of a ligand, which results in initiation of adipocyte differentiation. This differentiation process involves morphological changes, lipid accumulation and switching on of many adipocyte-specific genes such as GLUT4 and adiponectin (Rosen et al., 1999).

C/EBP or CCAAT Enhancer binding protein has six family members of which four (α,β,δ and ζ are expressed during adipogenesis. C/EBPβ and C/EBPδ are expressed early and are involved in the activation of PPARγ2 and C/EBPα, which acts later and is responsible for the expression of several adipocyte specific genes e.g. GLUT4, aP2 and UCP (Zuo et al., 2006).

ADD-1/SREBP-1 regulates the expression of enzymes responsible for FA synthesis and uptake and cholesterol synthesis (Kim and Spiegelman, 1996).

1.6 Membrane Trafficking

Trafficking of proteins in the endocytic and exocytic or secretory pathways involves many different steps; delivery of newly synthesised proteins to cell surface or other target organelles, internalisation of materials at the cell surface, transport within the cell and the maintenance of the membranes involved in protein movement around the cell. This is achieved by compartmentalisation of functions within distinct membrane bound organelles and transport vesicles that move membrane-bound and soluble proteins through the secretory pathway. At the various stages of the pathway, there are mechanisms in place to ensure that each protein is delivered, by the transport vesicle, to the correct destination.

1.6.1 The Secretory Pathway

Proteins synthesised in the cytosol may be destined for secretion outside the cell, insertion into the plasma membrane, or lysosomal degradation. Whatever their destination they must

reach it from their point of synthesis. In the classical secretory process, newly synthesised proteins are inserted into the ER and are subsequently transported to the Golgi apparatus where they are processed and packaged into vesicles for transport to the cell surface and fusion with the PM.

The sequence of events in the secretory pathway was first demonstrated in a set of experiments using pulse chase autoradiography on pancreatic acinar cells (Blobel and Dobberstein, 1975; Palade, 1975). By varying the pulse chase periods, it was shown that labelled proteins appeared first in the ER, then the Golgi and were then carried to the PM in secretory vesicles. The pathway was further elucidated in experiments in temperature-sensitive mutant yeast strains (Novick and Schekman, 1979). By studying strains of yeast that were deficient for the secretion of invertase at non-permissive temperatures, five stages were identified where secretion could be blocked. These were: transport into the ER form the cytosol, budding of vesicles from the ER, fusion of vesicles with the Golgi, transport to secretory vesicles from the Golgi and transport of vesicles to the cell surface, thus confirming the essential stages in the synthesis and export of secretory proteins.

1.6.1.1 Synthesis and Insertion into ER

Proteins destined for secretion are synthesised on ribosomes in the cytosol and transported co-translationally, either into the lumen of the ER in the case of soluble proteins (Rapoport et al., 1996) or into the membrane of the ER itself for membrane proteins (Mothes et al., 1997). Proteins are targeted to the ER by an N-terminal signal sequence that binds to the cytosolic signal recognition peptide SRP that in turn binds to the SRP receptor in the ER. When localised at the ER membrane, the growing polypeptide chain is either translocated into the ER through a pore shaped assembly of membrane proteins called the translocon, or in the case of membrane proteins, inserted in the membrane of the ER. The N-terminal signal sequence is subsequently cleaved by signal peptidase in the ER. The newly synthesised proteins are then exposed to the resident chaperones and enzymes in the lumen of the ER. Here they undergo various modifications, necessary for correct folding, such as N-linked glycosylation and disulphide bond formation, since only correctly folded proteins will leave the ER and be transported to the Golgi for transport to the cell surface or to lysosomes (Hammond and Helenius, 1994). The ER has several mechanisms for ensuring that misfolded or improperly assembled proteins are retained in the ER. Such proteins can be marked with an extra glucose at the end of the N-linked sugar chain. This causes them to be bound to chaperones such as calnexin and calreticulin resulting in retention in the ER until they are correctly folded (Oliver et al., 1997). Incorrectly folded or assembled

peptides can be transported back through he translocon into the cytosol (Plemper et al., 1997) where they are ubiquitinated and thus targeted to the lysosome for degradation. Correctly folded proteins are no longer retained by the chaperones and may leave the ER for the Golgi complex.

1.6.1.2 ER to Golgi Transport

Proteins leaving the ER travel to the Golgi in special transport vesicles. These vesicles budding from the ER are coated with the coat protein COPII, made from a 700kD complex of Sec31 and Sec13, a 400kD complex of Sec23 and Sec24 and the GTPase Sar1p. COPII vesicles form when Sec12p catalyses the exchange of GDP to GTP on Sar1p thus allowing it to bind to the ER membrane. Sar1pGTP in turn recruits Sec23/Sec24 and Sec13/ Sec31 (Barlowe et al., 1994). This coming together of the constituent components initiates polymerisation of the coat proteins and budding of the membrane, resulting in vesicle formation. Sec 16 is also required for the formation of ER to Golgi transport vesicles, possibly acting as a scaffold on which the COPII protein may assemble. After budding off from the ER the COPII vesicles lose their coats when Sec 23p (the GAP for the GTPase Sar1p) increases the rate of hydrolysis of the GTP bound to Sar1p, causing dissociation of the coat. The uncoated vesicles then fuse, forming vesicular-tubular clusters, which are thought to fuse forming the outer-most or cis-face of the Golgi.

Whilst secretory proteins are transported to the Golgi, resident enzymes and chaperones must remain in the ER and this is achieved by a retention sequence at their COOH terminal. This sequence of four amino acids Lys-Asp-Glu-Leu, or the KDEL sequence, binds to an integral membrane receptor in the cis-Golgi and the protein is then packaged into COPI coated vesicles and returned to the ER (Wilson et al., 1993). Membrane components from the ER, included in the transport vesicle must also be returned in order to maintain the ER membrane (Cosson and Letourneur, 1997) . These vesicles form when the small GTPase ARF1 GTP interacts with coatomer proteins αCOP and β COP, recruiting them to the membrane (Spang et al., 1998). It is thought that clustering of Arf1 causes the membrane to deform resulting in budding and formation of a vesicle.

1.6.1.3 The Golgi Complex

The N-linked oligosaccharides that are added to proteins in the ER are often initially trimmed by the removal of mannoses, and further sugars are added.

The Golgi complex comprises a set of stacked flat membrane bound cisternae. Each stack has a cis and trans face, the cis face being the entry face where proteins arriving from the ER are incorporated and the trans face being the exit face where proteins leaving the Golgi are packaged into transport vesicles. The Golgi compartments are also defined by their modifying enzymes e.g. phosphatases in the cis-Golgi and galactosyltransferase in the trans Golgi. As proteins pass through the Golgi they are further processed in readiness for packaging into vesicles and transportation to their final destination (Warren and Malhotra, 1998).

1.6.1.4 Transport from TGN to lysosome

N-linked oligosaccharides that are added to proteins in the ER are further processed in the Golgi. Mannose groups are removed and further sugars added. An example of this is the addition of mannose-6-phosphate; a sugar that ensures the protein is directed to late endosomes. Any protein destined for the LE or lysosomes will have arrived from the ER with an N-linked oligosaccharide precursor already attached. In the cis Golgi one or more mannose residues are phosphorylated. Proteins containing mannose-6-phosphate bind to man-6-phosphate receptors in the TGN and are incorporated into clathrin-coated vesicles. After budding from the TGN the vesicles lose their clathrin coat and the uncoated vesicles fuse with the late endosomal compartment. Here the low pH of the environment causes the protein to become dissociated from the mannose-6- phosphate receptor. The receptor is recycled back to the Golgi for re-use, and the protein is transported to the lysosome for degradation (Munier-Lehmann et al., 1996).

1.6.1.5 Pathways of secretion and trafficking from TGN

Most soluble secretory proteins are carried to the cells surface in vesicles that bud from the TGN (Figure 1.3), in the classical ER/Golgi dependent secretory pathway. In constitutive secretion i.e. the secretion of integral membrane proteins and receptors and extracellular matrix proteins, secretory vesicles are transported directly to the PM or the organelle where they function. Alternatively, in certain cell types displaying regulated exocytosis, cargo is packaged into immature storage granules (ISGs). These ISGs mature into secretory granules that are transported to the cell surface, where their contents are released, only in response to a specific stimulus e.g. in the case of insulin secretion from pancreatic β-cells, or synaptic release from neurons (Arvan and Castle, 1998; Arvan and Halban, 2004). Clathrin-coated vesicles bus from the TGN to transport material to the endosomal/lysosomal system. Some secreted proteins exhibit "constitutive-like" secretion and traffic to the cell surface via the endosomal system (Millar et al., 2000; Feng and

Arvan, 2003). There is also evidence that some proteins are exported from the cell independently of the classical secretory pathway. Interleukin 1β, galectin and FGF are examples of secreted proteins that lack a conventional signal peptide and are not found associated with classical secretory organelles (Nickel, 2003). Il-1β, along with several other cytokines, appears to translocate directly from the cytoplasm into an endolysosomal compartment that eventually fuses with the PM (Andrei et al., 1999). The pro-angiogenic growth factors FGF-1 and FGF-2 are thought to be translocated directly from the cytoplasm across the PM (Mignatti et al., 1992). The galectins-1 and 3, members of a family of β-galactoside-specific lectins of the extracellular matrix, accumulate directly below the PM where they are packaged into small membrane-bound vesicles called exosomes for transport to the extracellular space (Cooper and Barondes, 1990).

1.6.1.6 Coat Proteins and vesicle budding

There are three distinct coat proteins associated with intracellular transport, namely COPI, COPII and Clathrin. These coat proteins mediate budding events, forming vesicles and making sure the correct cargo is packaged (Cosson and Letourneur, 1997). Clathrin coated vesicles bud from the TGN and endosomes, and allow for the endocytosis of proteins at the PM (Crowther and Pearse, 1981). Individual molecules of clathrin self-assemble into a lattice around vesicles, as they bud from the membrane. Each clathrin molecule has an N-terminal domain that binds to a subunit of an adaptor complex (AP). AP1 functions at the TGN along with GGAs (Golgi-localised, γ-ear containing, ARF-binding proteins) to tether clathrin toTGN membranes. AP2 is involved in traffic from the plasma membrane, AP3 in trafficking to lysosomes and AP4 is found on vesicles in the TGN region (Robinson and Bonifacino, 2001). Adaptor complexes have subunits that are involved in cargo selection. Whether the coat protein is coatomer (COP) or clathrin, polymerisation of coat proteins is necessary for two important features of transport vesicles: concentration of cargo within the vesicle and curvature of the membrane leading to budding of the vesicle. COP and clathrin coated vesicles both shed their protein coat after budding before fusion with their target membrane.

1.6.1.7 Fusion with plasma membrane and SNARE hypothesis

Targeting and fusion of vesicles with the appropriate target membrane requires the interaction of membrane proteins called SNARES (soluble NSF-attachment protein receptors) (Sollner et al., 1993; Rothman, 1994). SNARES are present on vesicles (v-SNARES) and target membranes (t-SNARES) and form a stable complex, called the trans-

SNARE complex, when three α-helices from the t-SNARE and one from the v-SNARE assemble to make a structure of four parallel α-helices that bridges the two membranes (Sutton et al., 1998). Fusion of the membranes results in a cis-SNARE complex in which all the SNARES are associated with the same membrane. SNARES are disassembled by the chaperones NSF and SNAP and the SNARE proteins are recycled to be used in another round of vesicle fusion with its target membrane. At least 50 eukaryote SNARE proteins have been identified, each with a distinct localisation (Scales et al., 2000). It has however been demonstrated that the SNARES are not solely responsible for the specificity of the vesicle membrane interaction. Other proteins thought to be involved in determining the specificity of fusion reactions include tethering factors and the Rab family of GTPases. Tethering factors are also membrane specific, many are coiled-coil proteins that are predicted to assemble in a Rab-dependent manner to bring the two membranes together (Jahn et al., 2003).

1.6.1.8 The Exocyst complex

The Exocyst is a complex of eight proteins (Figure 1.4) that was first identified in budding yeast and is essential for exocytosis (Terbush et al., 1996). The proteins of the exocyst interact with one another to form a complex that is localised in the region of the PM (Grindstaff et al., 1998). The exocyst is involved in docking of secretory vesicles and is thought to have a role in guiding vesicles to fusion sites on the PM (Guo et al., 1999). Acting like a tethering factor, the exocyst complex is involved in the docking of exocytic vesicles with the PM. The exocyst complex is a necessary component in the docking of insulin vesicles at the PM in pancreatic β cells (Tsuboi et al., 2005) and is also required for the targeting of Glut4 vesicles to the PM during the process of insulin stimulated glucose transport (Inoue et al., 2003).

1.6.1.9 Arf and Rab GTPases in membrane transport

Two vital stages during membrane trafficking are regulated by small GTPases of the Arf and Rab families; these are formation and budding of transport vesicles and the delivery of transport vesicles to their target membrane respectively (Figure 1.5)

1.6.1.10 Rab

The Rab family of small GTPases are involved in the regulation of vesicle transport, conferring specificity to vesicle-membrane interactions. That Rab GTPases can regulate these interactions in a specific way depends on their ability to switch between the inactive,

cytosolic GDP-bound and the active, membrane localised GTP-bound forms (Figure 1.5). Newly synthesised and prenylated Rab is brought to the membrane by a Rab escort protein (REP). Here Rab-GDP is activated by a guanine nucleotide exchange factor (GEF) and, as Rab-GTP, is able to recruit effector proteins to the membrane. Rab-GTP is then hydrolysed to Rab-GDP with the help of a GTPase activating factor (GAP) rendering it susceptible to removal from the membrane by a Rab GDP dissociation factor (RabGDI). Rab-GDP and RabGDI form a complex in the cytosol and constitute a supply of Rab that can be recycled to the membrane and activated (Zerial and McBride, 2001).

More than 60 Rab proteins have been identified with each Rab being localised to a particular intracellular membrane. (Figure 1.6) Some facilitate fusion of vesicles with their target membrane by binding to effector molecules that may act as tethers. The specificity of membrane tethering is thought to depend on Rab effectors The tethering factor p115 was shown to be a rab effector that interacts with the SNARE complex (Allan et al., 2000).

1.6.1.11Arfs

Like the Rab proteins, ADP-ribosylation factors (Arfs) are GTP binding proteins that cycle between their GTP-bound and GDP-bound conformations. Their action is dependent on the binding and hydrolysis of GTP, and this is regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) respectively.

Arfs were originally classified into three groups, class I (Arf1, Arf2, and Arf3), class II (Arf4 and Arf5) and class III (Arf6) (Kahn et al., 1991). Activated Arf proteins act by modifying the lipid composition and recruiting coat proteins on to membranes, resulting in the formation of vesicles. The coat proteins include COP I, the clathrin adaptor protein AP1 and GGAs (Golgi associated α-adaptin homology ARF-binding proteins) (Robinson and Bonifacino, 2001; Bonifacino and Glick, 2004). Arfs regulate lipid-metabolising enzymes like Phospholipase D and phosphotidylinositol 4-phophate 5-kinase, resulting in the release of PIP_2 and leading to curvature of the membrane (Brown et al., 1993). Arfs1, 3and 5, when in their GTP-bound conformation, associate with Golgi membranes. Whereas Arf6 cycles between the PM and recycling endosomes (D'Souza-Schorey et al., 1998).

1.6.2 The Endocytic pathway

Molecules to be internalised bind to specific receptors on the plasma membrane. These receptor–ligand complexes are then concentrated in clathrin rich areas called coated pits. Polymerisation of clathrin molecules results in a concentration of sites available to bind the

cargo adaptor proteins that will in turn bind to cargo molecules. In this way, clathrin concentrates cargo into vesicles. During vesicle assembly at the plasma membrane the μ2 subunit of AP2 adaptor complex interacts directly with tyrosine and dileucine motifs on the cytoplasmic end of the receptor (Ohno et al., 1995). After budding, the vesicles lose their clathrin coat. These uncoated vesicles fuse to become early endosomes, and these mature further to form late endosomes (LE). What happens next depends on the type of receptor. Most receptors dissociate from their ligand in the low pH environment of the LE e.g. in the case of the LDL (low density lipoprotein) receptor. LDL is a complex that carries cholesterol in the bloodstream and is internalised after binding to LDL receptors on the cell surface. In the LE, the LDL dissociates from its receptor and the receptor is recycled back to the PM. The LDL is transported to the lysosome where lysosomal enzymes hydrolyse the LDL complex, releasing cholesterol (Brown and Goldstein, 1979)(Figure 1.7). Transferrin follows a similar pathway but also recycles it ligand (Hopkins, 1983). Transferrin bound to iron binds to the transferrin receptor TfnR and is internalised into the EE. Here the low pH causes the iron to leave the Tfn but the Tfn remains bound to its receptor and they are both recycled to the PM. Once they have reached the extracellular face, the Tfn dissociates from its receptor. On its way to the surface some Tfn is held in recycling endosomes which is distinct from late endosomes and lysosomes and whose pH is slightly higher than that of the EE (Yamashiro et al., 1984).

1.7 Aims of this study

The main aims of this study were to further investigate the mechanisms of secretion of ACRP30 in 3T3-L1 adipocytes and to analyse the secreted proteome of 3T3-1 adipocytes with a view to identifying novel secreted proteins.

To address some of the questions surrounding ACRP30 trafficking, I have investigated the involvement of subcellular organelles by disrupting trafficking through the Golgi and endosomes. I have also examined the involvement of the exocyst complex proteins Sec6 and Sec8 and syntaxins 8 and 12 in ACRP30 secretion, as well as the GTPases Arf6 and Rab11.

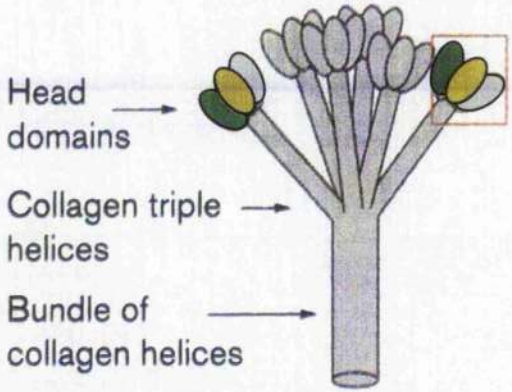
With the aim of identifying novel proteins secreted by 3T3-L1 adipocytes, I have analysed the secreted proteome of 3T3-L1 adipocytes by 2D LC-MS/MS. Furthermore I have investigated the functional roles of several proteins that were identified as potential novel adipokines.

Figure 1.1 Structure of ACRP30

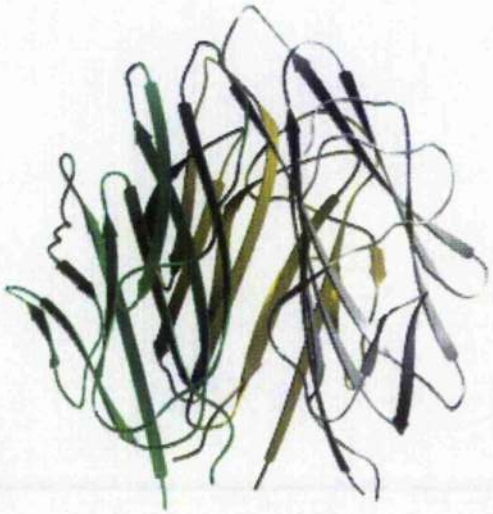
The crystal structure of the homotrimeric globular fraction of ACRP30 at a resolution of 2.1Å (Shapiro and Scherer, 1998).

Also shown is the crystal structure of TNF-α, which shows structural homology to ACRP30.


1
17
45
110
247
SS
NH
Collagen
Globular Domain


Head domains
Collagen triple helices
Bundle of collagen helices

ACRP30



TNF-α

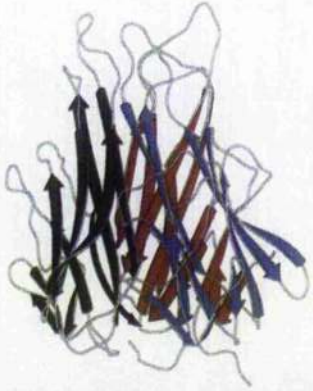


Figure1.2 Insulin signalling pathways

Insulin binding to the tyrosine kinase insulin receptor triggers the activation of intracellular signalling cascades. The Insulin receptor subunit (IRS), when phosphorylated becomes a docking site for PI 3-kinase, thus bringing about the phosphorylation of PtdIns(4,5)P_2 (PIP_2) by the p110 subunit of IRS and resulting in the activation of the PI 3-kinase cascade. (PIP_2) phosphorylates (PIP_3), which in turn phosphorylates and activates the phosphoinositide-dependent protein kinase 1 (PDK1), which is vital for activation of Akt, or protein kinase B. PKB/Akt and two atypical PKC isoforms ξ andλ are thought to be involved in translocation of the insulin dependent glucose transporter GLUT4 to the cell surface (Lizcano and Alessi, 2002).

On translocation to the plasma membrane GLUT4 causes the cell to take up glucose from the extracellular environment.

Insulin binding to the insulin receptor may also initiate phosphorylation of the proto-oncogene Cbl. The phosphorylated Cbl, in complex with CAP,

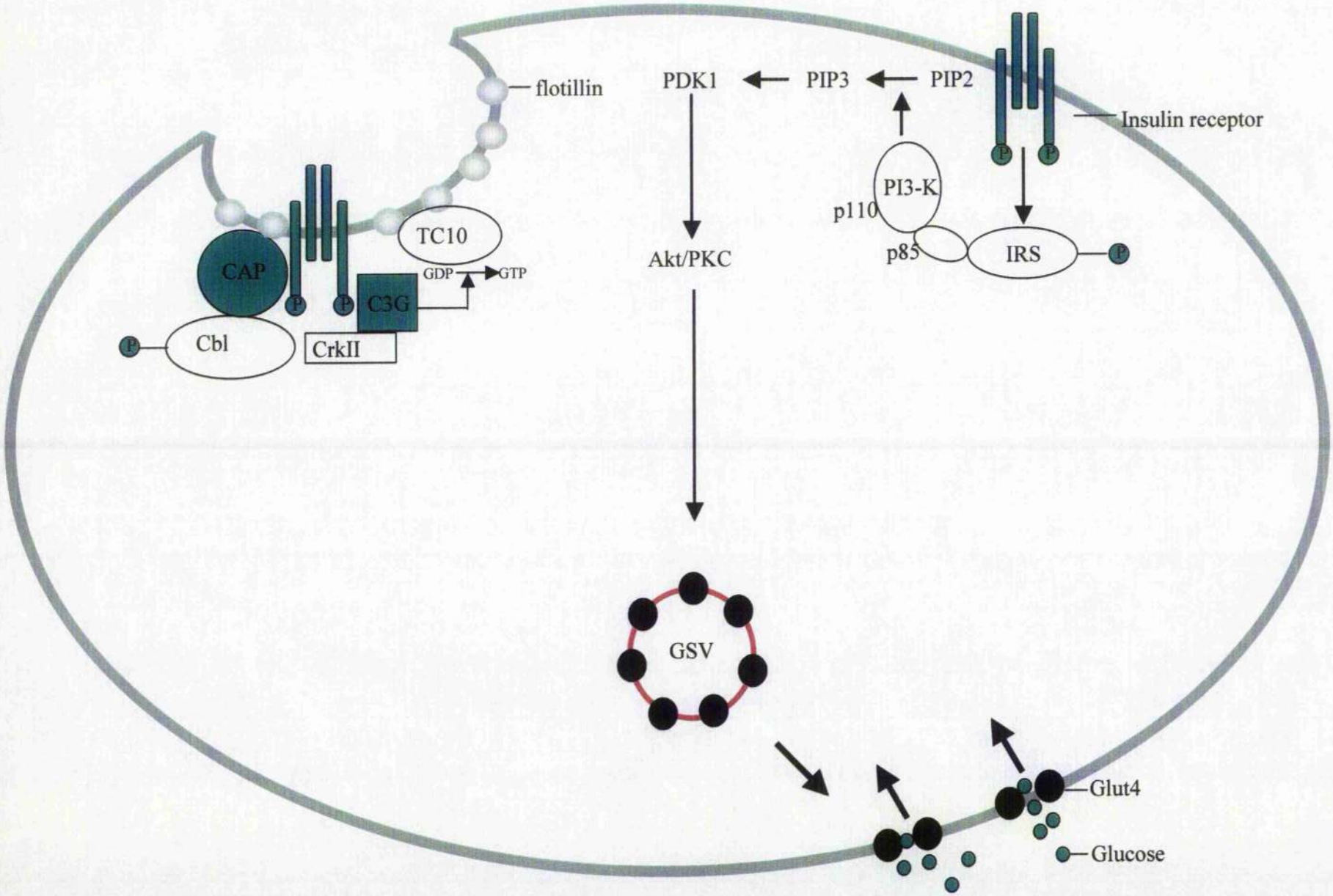

flotillin
PDK1
PIP3
PIP2
Insulin receptor
PI3-K
p110
p85
IRS
TC10
CAP
GDP
GTP
C3G
Cbl
CrkII
Akt/PKC
GSV
Glut4
Glucose

Figure 1.3 Pathways of Secretion

Most soluble secretory proteins are carried to the cells surface in vesicles that bud from the TGN, in the classical ER/Golgi dependent secretory pathway. In constitutive secretion secretory vesicles are transported directly to the PM or their target organelle. In certain cell types displaying regulated exocytosis, cargo is packaged into immature storage granules (ISGs). These ISGs mature into secretory granules that are transported to the cell surface, where their contents are released, only in response to a specific stimulus e.g. in the case of insulin secretion from pancreatic β-cells, or synaptic release from neurons. Clathrin-coated vesicles bus from the TGN to transport material to the endosomal/lysosomal system. Some secreted proteins exhibit "constitutive-like" secretion and traffic to the cell surface via the endosomal system. There is also evidence that some proteins are exported from the cell independently of the classical secretory pathway. This "non-classical secretion" may take several forms: translocation to the PM directly from an endolysosomal compartment; translocation from the cytoplasm across the PM and transport across the PM in small, membrane-bound exosomes.

Key to Figure 1.3


Undefined membrane transporter


Regulated secreteory product

Mannose-6-phosphate receptor

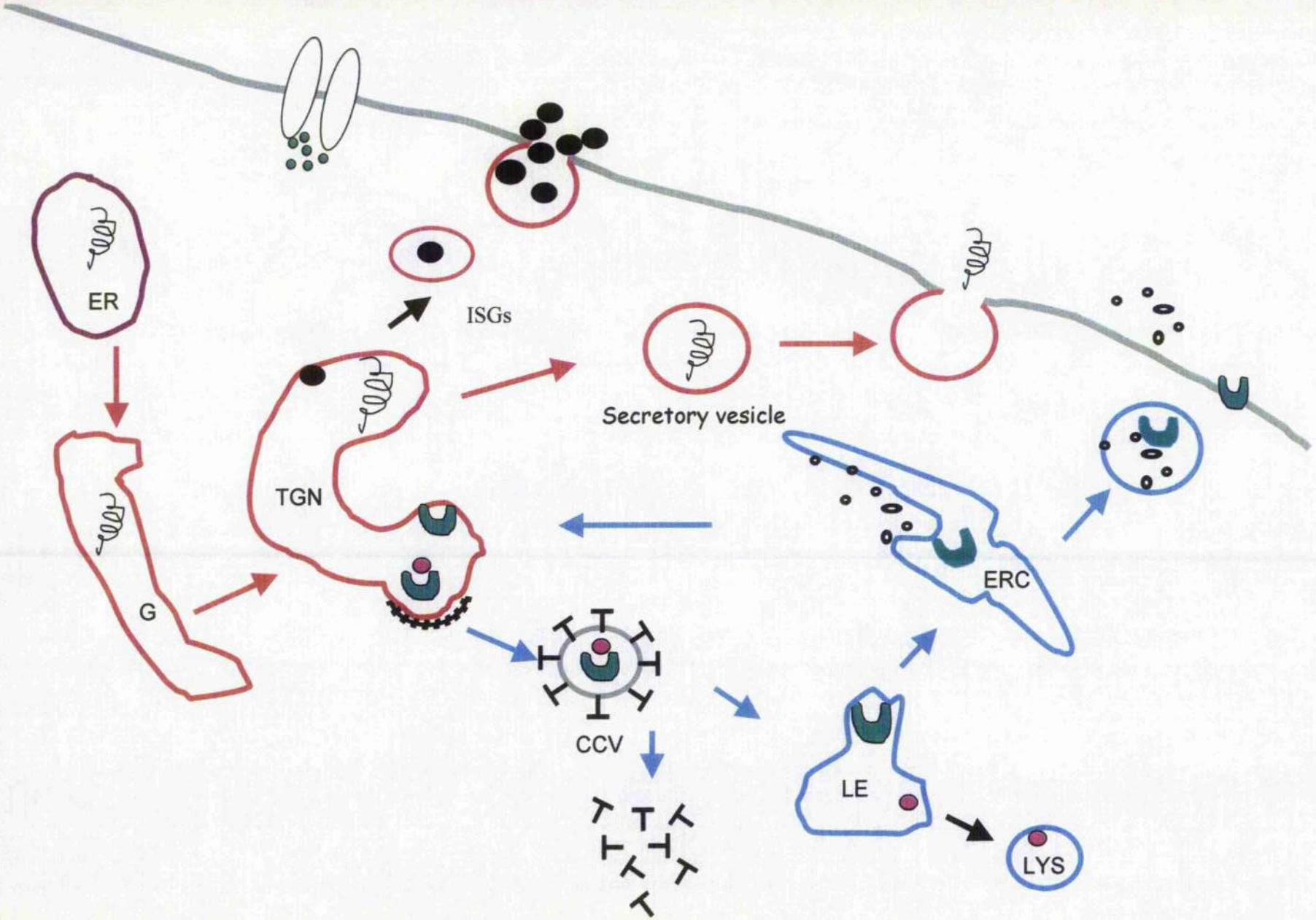

Mannose-6-phosphate


FGF


Constitutively secreted protein

IL-1

Clathrin



Red arrows show constitutive secretory pathway, Black arrows show regulated secretion from ISGs, Blue arrows show recycling of receptors, and secretion through endosomes.

Figure 1.4 The Exocyst

Figure 1.4 shows the eight protein-subunits that make up the exocyst. Several of these are known to be effectors of GTPases: Sec4, Rho1, Cdc42 and Ral1 all interact with exocyst proteins.

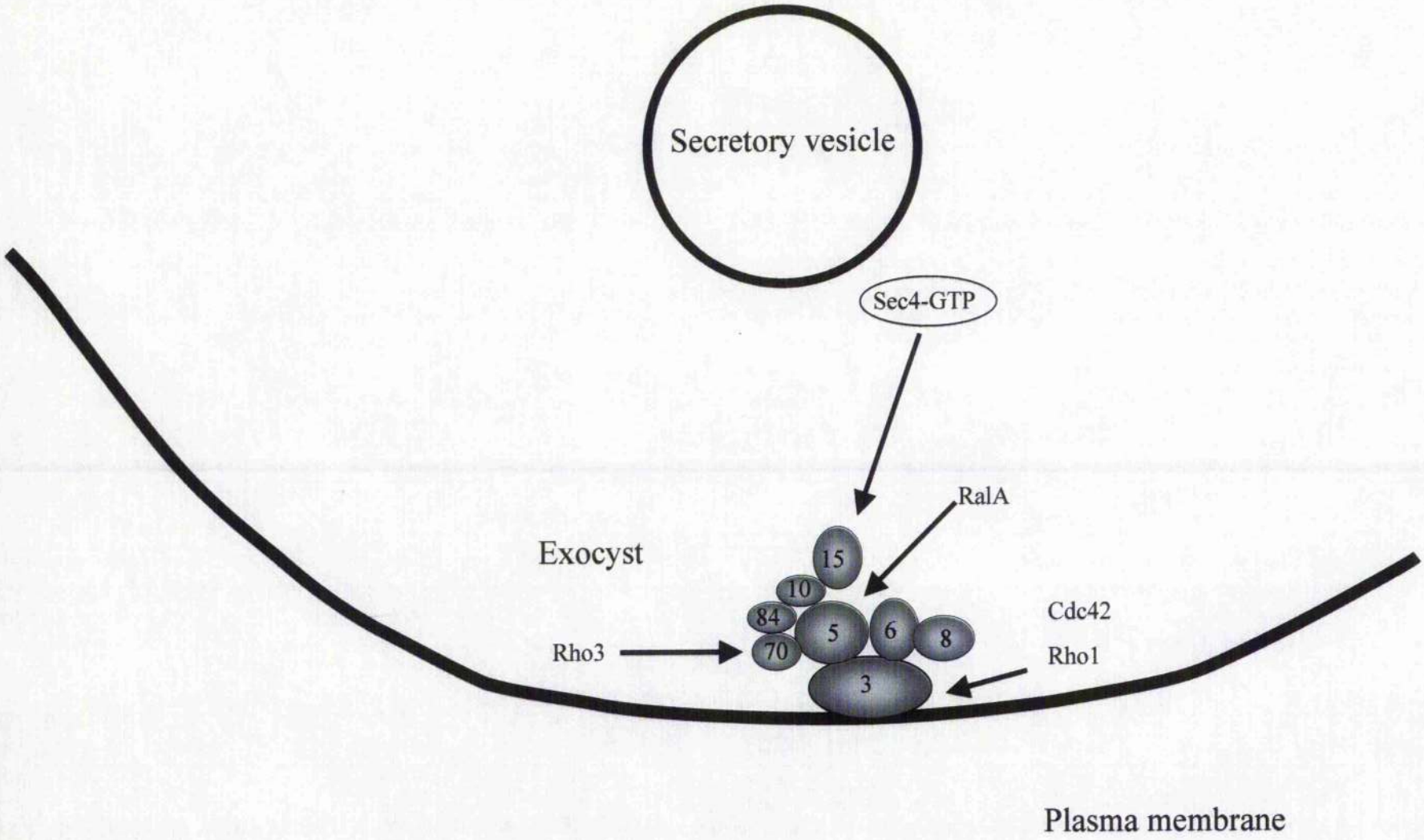

Secretory vesicle
Sec4-GTP
RalA
Exocyst
15
10
84
5
6
8
70
3
Rho3
Cdc42
Rho1
Plasma membrane

Figure 1.5 The GTP/GDP cycle of Rab proteins

Newly synthesised and prenylated Rab is brought to the membrane by a Rab escort protein (REP). Here Rab-GDP is activated by a guanine nucleotide exchange factor (GEF) and, as Rab-GTP, is able to recruit effector proteins to the membrane. Rab-GTP is then hydrolysed to Rab-GDP with the help of a GTPase activating factor (GAP) rendering it susceptible to removal from the membrane by a Rab GDP dissociation factor (RabGDI). Rab-GDP and RabGDI form a complex in the cytosol and constitute a supply of Rab that can be recycled to the membrane and activated.

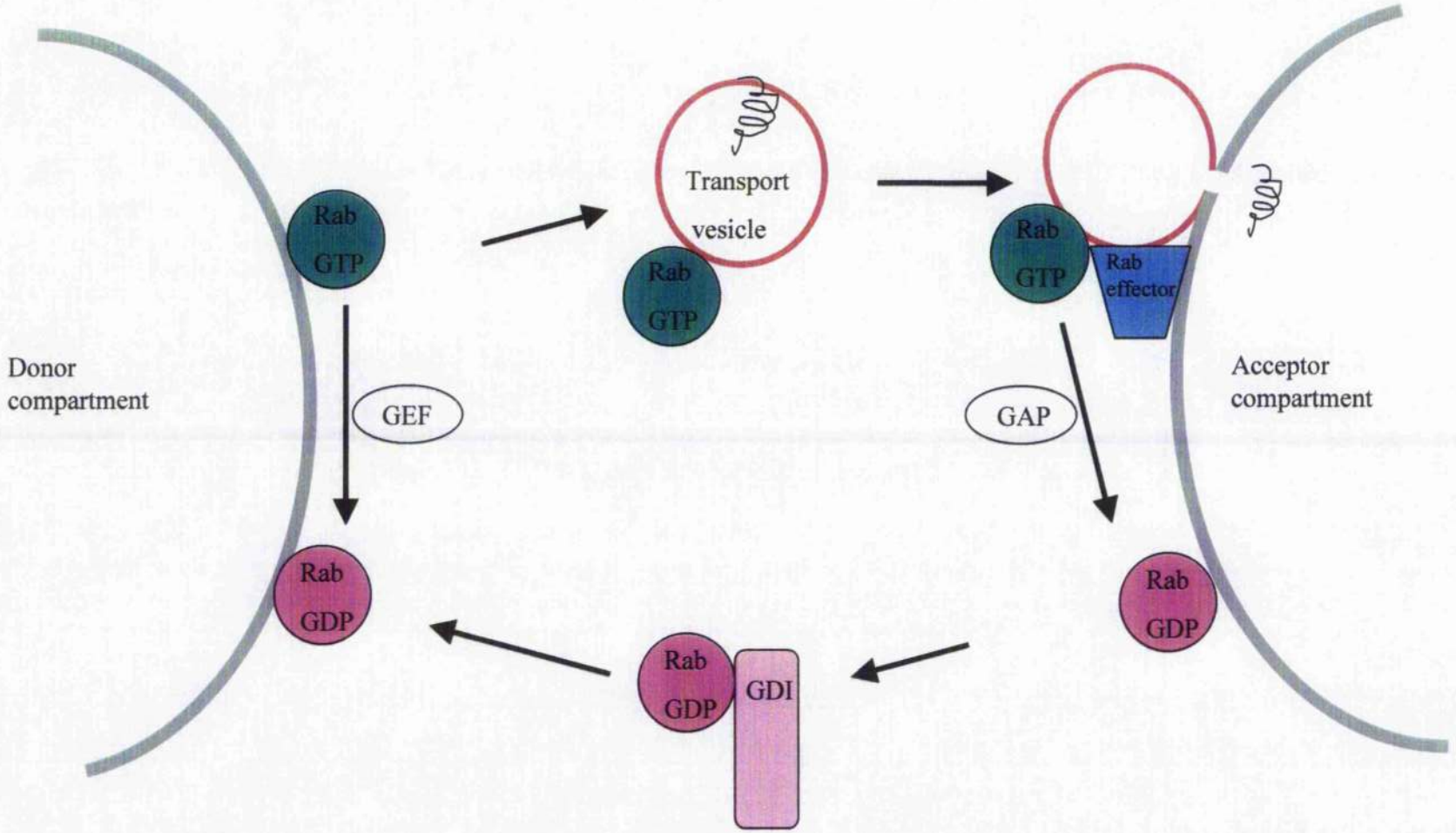

Donor
compartment
Rab
GTP
GEF
Rab
GDP
Transport
vesicle
Rab
GTP
Rab
GTP
Rab
effector
GAP
Acceptor
compartment
Rab
GDP
Rab
GDP
GDI

Figure 1.6 Intracellular distribution of Rab proteins

More than 60 Rab proteins have been identified, each one being associated with a distinct membrane in the cell. Figure 1.6 shows the localisation of some of these Rabs.

Rab	Localisation
Rab1	Golgi network
Rab2	ER → cis-Golgi network
Rab3	Secretory vesicles
Rab5	PM →Early endosomes, Early endosomes→ Late endosomes
Rab7	Early endosomes →Late endosomes
Rab8	Constitutive secretory vesicles
Rab11	Recycling endosomes

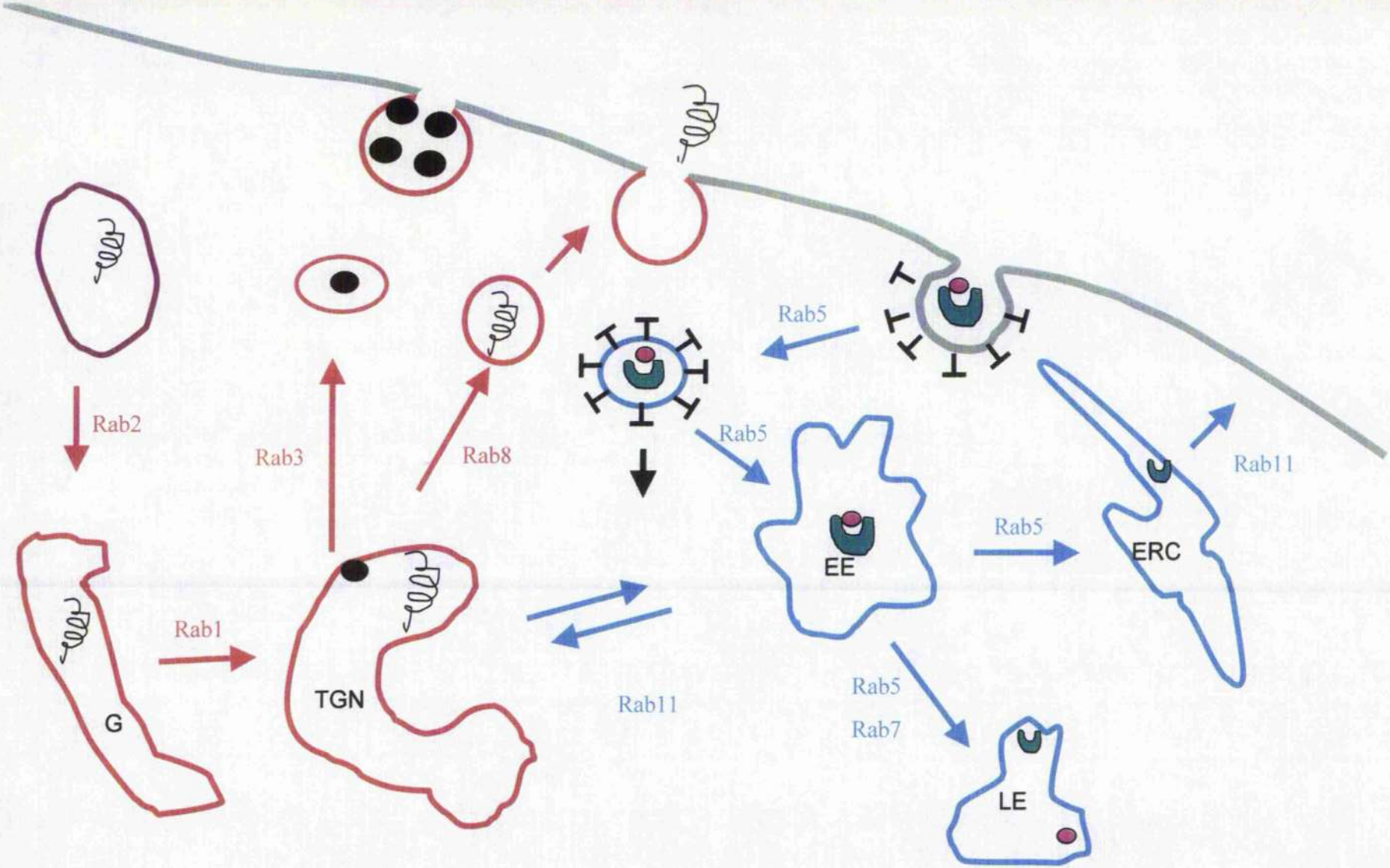
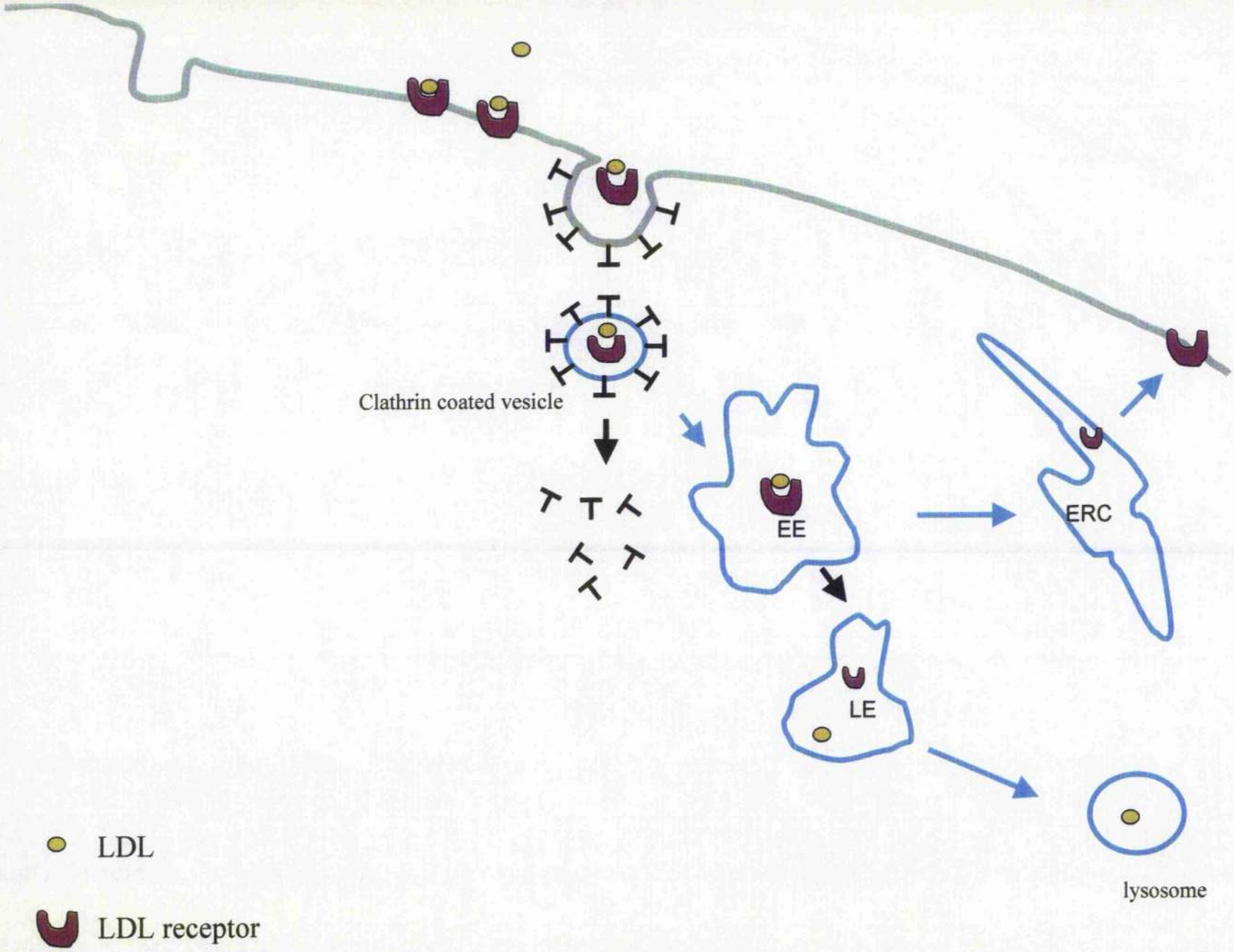

Rab2
Rab3
Rab8
Rab5
Rab5
Rab1
Rab11
Rab5
Rab11
Rab5
Rab7
G
TGN
EE
ERC
LE

Figure 1.7 Endocytosis

Molecules to be internalised bind to specific receptors on the plasma membrane. These receptor–ligand complexes are then concentrated in clathrin rich areas called coated pits. Polymerisation of clathrin molecules results in a concentration of sites available to bind the cargo adaptor proteins that will in turn bind to cargo molecules. In this way clathrin concentrates cargo into vesicles. After budding, the vesicles lose their clathrin coat. These uncoated vesicles fuse to become early endosomes, these mature further to form late endosomes (LE). The LDL (low density lipoprotein) receptor dissociates from its ligand (LDL) in the low pH environment of the LE. The LDL receptor is recycled back to the PM via the endosomes and the LDL is hydrolysed in the lysosome.


Clathrin coated vesicle
EE
ERC
LE
lysosome
LDL
LDL receptor

Chapter 2 Materials and Methods

2.1 Materials

All materials and reagents used were of the highest quality and were obtained from the following suppliers:

2.1.1 General Reagents

Anachem Ltd., Luton, Bedfordshire, UK

Acrylamide solution 30% (w/v) (ratio of 37.5:1 acrylamide:bis-acrylamide)

Bio-Rad Laboratories Ltd., Hemel Hempsted, Hertfordshire

Bio-Rad protein assay

BDH Ltd., Poole, Dorset, UK

Calcium chloride

Disodium hydrogen orthophosphate

Sodium chloride

Sodium dihydrogen orthophosphate dihydrate

Sucrose

Calbiochem, Merck Chemicals Ltd.,Padge Road, Beeston, Nottingham NG9 2JR, UK

Polymyxin B sulphate

Fisher Ltd., Loughborough, Leicestershire, UK

Acetone

Ammonium persulphate

Ethanol

Ethylenediaminetetraacetic acid, disodium salt (EDTA)

Glucose

Glycine

N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)

Hydrochloric acid

Magnesium sulphate

Methanol

Potassium chloride

Potassium dihydrogen orthophosphate

Sodium dodecyl sulphate

Tris Base

Kodak Ltd., Hemel Hempsted, Hertfordshire, UK

X-Omat S film

McQuilkin &Co, East Kilbride, UK

Marvel powdered milk

New England Biolabs (UK) Ltd., Hitchin, Hertfordshire, UK

Prestained protein markers, broad range (6-175 kD)

Schleicher and Schuell, Dassel, Germany

Nitrocellulose membrane (pore size 0.45 μm)

Whatman International Ltd., Maidstone, Kent, UK

Whatman No.1 filter paper

Whatman No.3 filter paper

NEN Dupont (UK) Ltd., Stevenage, Hertfordshire, UK

[^{3}H] 2-deoxy-D-glucose

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete protease inhibitor cocktail tablets

All other general reagents were purchased from Sigma Chemical Company Ltd., Poole, Dorset, UK., unless stated otherwise.

2.1.2 Cell culture Reagents and Materials

American Type Culture Collection

3T3-L1 Fibroblasts

Invitrogen Gibco, Paisley, UK

Dulbecco's modified Eagle's medium (without sodium pyruvate, with 4500mg/L glucose)

Dulbecco's modified Eagle's medium (without sodium pyruvate, with 4500mg/L glucose, without phenol red)

Foetal calf serum

Newborn calf serum

0.05% Trypsin/EDTA solution

Penicillin/Streptomycin solution comprising 10,000 units/mL penicillin and 10,000μg/mL streptomycin sulphate in 0.85% saline.

200mm (100X) L-Glutamine solution

BD Biosciences

10cm culture dishes

12 well culture plates

24 well culture plates

Sterile plastic pipettes

Costar

25cm^2, 75 cm^2 and 150 cm^2 culture flasks

TCS Cellworks Ltd., Park Leys, Botolph Claydon, Buckinghamshire, MK18 2LR, UK

Normal Human Umbilical Vein Endothelial Cells (pooled)

Large Vessel Endothelial Cell Basal Medium

Large Vessel Endothelial Cell Growth Supplement

Antibiotic Supplement

2.1.3 Antibodies

Ambion (Europe) Ltd., Huntingdon, Cambridgeshire, UK

Mouse monoclonal anti-GAPDH IgG_1, raised against purified rabbit muscle GAPDH

(Cat # 4300)

Amersham International Plc, Aylesbury, Buckinghamshire, UK

Horseradish Peroxidase (HRP)-conjugated donkey anti-rabbit IgG (whole molecule) (Cat # NA934V)

Horseradish Peroxidase (HRP)-conjugated sheep anti-mouse IgG (whole molecule) (Cat # NA934V)

Horseradish Peroxidase (HRP)-conjugated goat anti-rat IgG (whole molecule) (Cat # NA931)

BD Biosciences

Mouse anti-Nm23 monoclonal IgG_{2b}, generated from human Nm23 (Cat # 610247)

Sigma, Poole, Dorset

Horseradish Peroxidase (HRP)-conjugated rabbit anti-goat IgG (whole molecule) (Cat # A5420)

Promega

Horseradish Peroxidase (HRP)-conjugated rabbit anti-chicken IgY (Cat # G1351)

MBL Ltd., Naka-ku Nagoya, Japan

Rabbit anti-Hsp70 (Hsp72) whole rabbit serum, raised against recombinant human Hsp70 (Cat # SR-B812)

Rat monoclonal anti-Hsp90α IgG_{2a}, clone 9D2, raised against purified human Hsp90 (Cat # SR-B840)

R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK

Goat anti-mouse CXCL4/PF4 IgG, raised against purified, *E. Coli*-derived, recombinant mouse CXCL4 (Cat # AF595)

Santa Cruz Biotechnology Inc.,(Autogen Bioclear, Calne, Wiltshire, UK)

PPARγ (E-8) mouse monoclonal IgG_1, raised against a sequence mapping at the C-terminus of PPARγ of human origin (identical to corresponding mouse sequence) (Cat # sc-7273)

Rabbit anti-C/EBPα IgG, raised against a peptide mapping within an internal region of C/EBPα (Cat # sc-61)

Rabbit anti c-myc IgG, raised against a peptide mapping near the C-terminus of human c-myc (Cat # sc-789),

Gentaur BVBA, Brussels, Belgium

Chicken anti-orosomucoid IgY, raised against purified human plasma protein (Cat # A22927F)

Anti-adipsin anti-serum was generously provided by Professor Bruce Spiegelman (Dana Farber Cancer Institute, Boston) (Kitagawa et al., 1989)

Adenovirus

Rab11-S25N was kindly donated by Rytis Prekeris, University of Colorado Health Sciences Centre, Denver, Colorado (Hickson et al., 2003).

Fast-cycling Arf6 mutant was kindly donated by Lorraine Santy, Eberly College of Science, Penn State University, PA, USA (Santy, 2002).

2.1.4 Recombinant and Purified Proteins

Abnova Corporation, Taipei, Taiwan

Full length recombinant Leucine-rich alpha-2-glycoprotein 1. Produced by *in vitro* translation in a cell-free wheat germ system.

Full length recombinant Nucleoside diphosphate kinase A (Nm23). Produced by *in vitro* translation in a cell-free wheat germ system.

MBL Ltd., Naka-ku Nagoya, Japan

Recombinant HSP70. This protein was purified from an *E. Coli* protein expression system.

Recombinant HSP90α. This protein was cloned from a HeLa cDNA library and expressed in *E. Coli*

R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK

Recombinant CXCL4/ Platelet Factor 4. A DNA sequence encoding mature mouse PF4 was expressed in *E.Coli*.Endotoxin: <1.0 EU per 1μg as determined by the LAL method

Scipac Ltd., Sittingbourne, Kent

Orosomucoid purified from pooled normal human serum

Sigma

Human recombinant Tumour Necrosis Factor-α, expressed in yeast.

Endotoxin: <10EU/mL by LAL test

2.2 Buffers

2.2.1 General Buffers

Phosphate Buffered Saline (PBS)

137mM NaCl, 10 mM Na_2HPO_4, 2.6mM KCl, 1.7mM KH_2PO_4 (pH 7.4)

Krebs Ringer Phosphate Buffer (KRP)

64mM NaCl, 2.5mM KCl, 2.5mM $NaH_2PO_4.2H_2O$, 0.6mM $MgSO_4.7H_2O$, 0.6mM $CaCl_2$ (pH7.4)

HES Buffer

255mM sucrose, 20 mM HEPES, 1mM EDTA (pH 7.4)

SDS Lysis Buffer

20 mM Tris, 0.05% SDS, protease inhibitors

Sucrose Gradient Buffer

20mM HEPES, 100mM NaCl, 1mM EDTA (pH 7.4)

2.2.2 Electrophoresis Buffers

SDS-PAGE Sample Buffer

93mM Tris-HCl pH6.8, 10% (w/v) glycerol, 2%(w/v) SDS, 0.01mg/mL bromophenol blue, 20 mM dithiothreitol (added immediately before use)

Electrode Buffer

192mM Tris, 40 mM Glycine, 0.1% (w/v) SDS pH 8.3

Transfer Buffer for Western blotting

192mM Tris, 40 mM Glycine, 10% (v/v) MeOH pH8.5

2.3 Stains

Coomassie Brilliant Blue Stain

0.25% Brilliant Blue R dissolved in a mixture of 10% (v/v) glacial acetic acid, 45% (v/v) MeOH, 45% dH_2O

Coomassie Destain

10% glacial acetic acid, 45% (v/v) MeOH, 45% dH_2O

Oil Red O stain

A stock of Oil Red O stain was prepared by dissolving 0.7 g Oil Red O in 200 mL isopropanol and stirring at room temperature overnight. This stock was filtered through a 0.2 μm filter and stored at 4°C.

To prepare a working solution, 6 parts Oil Red O stock was mixed with 4 parts dH_2O. This was allowed to stand at room temperature for 20 min before being filtered through a 0.2 μm filter.

2.4 Cell Culture Methods

2.4.1 Culture of 3T3-L1 adipocytes

3T3-L1 fibroblasts were cultured in 75 cm^2 flasks, using DMEM culture medium containing 10% (v/v) newborn calf serum and 1% (v/v) penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 10% (v/v) CO_2. The culture medium was replaced every 48 h. When sub-confluent the cells were sub-cultured by trypsinisation. Following aspiration of medium, the cells were washed 1X with 3 mL trypsin/EDTA solution, after which cells were incubated at 37°C with 2 mL trypsin/EDTA solution until all cells were detached from the surface of the flask. Following re-suspension in 100 mL DMEM containing 10% (v/v) NCS, cells were re-distributed to fresh 75 cm^2 flasks, 10 cm culture plates or 12-well culture plates.

2.4.2 Differentiaton of 3T3-L1 adipocytes

Cells were differentiated on day 4 post-confluence by the addition of DMEM containing 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin solution, 0.25μM dexamethasone, 0.5mM methyl isobutylxanthine and 1μg/mL insulin. This medium was prepared using a stock of 2.5mM dexamethasone in ethanol, a 500X stock solution of methyl isobutylxanthine, which was prepared in 1M KOH, and a 1mg/mL stock of insulin in 0.01M HCl.

48h later the differentiation medium was replaced with DMEM containing 10% FCS, 1% penicillin and streptomycin and 1μg/mL insulin. Following differentiation the medium was replaced every 48h with DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were used 8-14 days post-differentiation.

2.4.3 Freezing and storage of 3T3-L1 cells

Following incubation at 37°C with trypsin, cells from one 75 cm^2 flask of 3T3-L1 cells were re-suspended in 3 mL of DMEM culture medium containing 10% (v/v) newborn calf serum and 1% (v/v) penicillin and streptomycin solution, and the cell suspension was centrifuged at 2000 x *g* for 4 min. The supernatant was removed by aspiration and the cell pellet was gently agitated and re-suspended in 2mL of DMEM containing 20% (v/v) NCS and 10% (v/v) DMSO. The suspension was divided between two 1.8 mL polypropylene cryovials and placed in a Styrofoam box at -80°C overnight before being transferred to liquid nitrogen for long-term storage.

2.4.4 Thawing of frozen stocks from liquid nitrogen

A vial of cells was removed from liquid nitrogen and thawed in a 37°C waterbath. The cell suspension was pipetted into a 75 cm^2 flask and 15 mL of pre-warmed growth medium was slowly added to the cells. The medium was replaced 24 h later.

2.4.5 Culture, freezing and storage of U937 cells

U937 cells were grown as a suspension culture in 75cm^2 flasks in RPMI 1640 medium containing 10% (v/v) FCS and 1% (v/v) Penicillin/streptomycin. The cells were subcultured every 48h, by removing 50% of the culture and replacing it with an equivalent volume of fresh growth medium. The volume of the culture was never allowed to exceed 50mL.

The contents of one 75 cm^2 flask of U937 cells in the log phase of growth were transferred to a sterile centrifuge tube and centrifuged at 2000 x *g* for 4 min. The supernatant was aspirated and the cell pellet was gently agitated and re-suspended in 5 mL of 90% (v/v) FCS, 10% (v/v) DMSO. The cell suspension was divided between five 1.8 mL polypropylene cryovials and placed in a Styrofoam box at -80°C overnight before being transferred to liquid nitrogen for long-term storage.

2.4.6 Culture of HEK 293 cells

HEK 293 cells were routinely grown in DMEM containing 5% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin solution and 1% (v/v) L-Glutamine.

2.4.7 Culture of HUVECS

Cryopreserved HUVECS were purchased from TCS Cellworks, Park Leys, Botolph Claydon, Buckinghamshire, MK18 2LR, UK. Cells were thawed and seeded at approximately 2,500 viable cells per cm^2 in Costar 25cm^2 flasks, in TCS Large Vessel Endothelial Cell Medium. Medium was prepared by adding 10mL TCS Large Vessel Endothelial Cell Growth Supplement and 0.5mL antibiotic supplement to 500mL TCS Large Vessel Endothelial Cell Basal Medium. Cells were refed with fresh growth medium every 48h and subcultured when the cells reached 60-80% confluence. HUVECS were used for experiments between passages 2 and 6.

2.4.8 3T3-L1 Differentiation assay

3T3-L1 cells were grown to confluence in 12-well culture plates, and 4 days post confluence the differentiation protocol was started. Cells were treated with various recombinant or purified proteins at different concentrations, and these proteins were added at the same time as the differentiation medium (day 0 of differentiation) and subsequently on days 2, 4 and 6 of differentiation. Differentiation was measured by uptake of Oil Red O stain (see section 2.12)

2.4.9 Monocyte adhesion assay

10^4 HUVECs were seeded into each well of a 24-well plate and grown at 37°C for 48 h. The cells were then incubated for 4 h with the treatments indicated. Each condition was repeated in 4 separate wells within one experiment, and each experiment carried out three times. After incubating with each condition the medium was aspirated, the cells washed 2X with serum free HUVEC medium and the cells overlaid with $1X10^5$ U937 promonocytic cells per well in serum-free HUVEC medium. The cells were allowed to adhere for 1 h at 37°C before they were removed and the monolayers were washed 3X with serum free DMEM. The cells were then fixed in 0.5 mL per well of PBS pH 7.2 containing 4% (v/v) formaldehyde and 5% (w/v) sucrose. The number of U937 cells adhering to HUVECs was analysed by phase contrast microscopy. The analysis was carried out by counting 3

separate fields from each well, making a total of 12 counts per condition per experiment. These 12 counts were averaged to give a result for each condition per experiment. This result was expressed as a percentage of the control (no treatment) and the results from three separate experiments were averaged. The results from one experiment were independently counted by a colleague, and the same results were obtained. To minimise the possible effects of any contaminating endotoxins from the recombinant proteins, all recombinant proteins were incubated for 30m with 2μg/mL Polymyxin B sulphate, which binds to and inactivates endotoxins.

2.4.10 Adenoviral infection of 3T3-L1 adipocytes

3T3-L1 were routinely infected on day 8 of differentiation, unless stated otherwise. Cells were washed in serum-free DMEM and then incubated overnight in 0.5 mL per well of a 12 well plate of serum-free DMEM containing virus at an m.o.i of 50 ifu/cell. The virus-containing medium was aspirated and replaced with DMEM containing 10% fetal bovine serum, and the cells were used between 24 h and 72 h later, depending on the experiment.

2.5 3T3-L1 Secretion Assay

3T3-L1 adipocytes were cultured on 10 cm culture dishes or 12 well culture plates. Cells were used for secretion assays when at day 8-11 post-differentiation, where day 0 is equivalent to the day of addition of IBMX, dexamethasone and insulin. Cells were incubated at 37°C in serum free DMEM growth medium for two hours, before being washed 2X with Krebs Ringer Phosphate (KRP) buffer (see Section 2.2.1). Thereafter, cells were incubated with 10 mL (for 10 cm dishes) or 0.5 mL (in the case of 12 well plates) KRP ± 1μM Insulin at 37°C. The KRP was then collected from the cells at defined times, and the secreted proteins were TCA precipitated from the KRP buffer.

Where the assay was carried out in 24 well plates the TCA-precipitated pellet was resuspended in 50μL 1 X SDS-PAGE sample buffer (section 2.2.2) and 20μL of this was loaded onto a 12% SDS-PAGE protein gel (section 2.13). Separated proteins were electrophoretically transferred to nitrocellulose membrane at 90mA overnight, blocked with PBS containing 5% Marvel and then incubated in primary antibody or pre-immune serum diluted 1:2000 in PBS containing 1% Marvel for 1h at room temperature. After incubation membranes were washed extensively over a period of 20min with PBS and incubated with horseradish-peroxidase labelled anti-rabbit IgG. Proteins were visualised using enhanced chemiluminescence (section 2.15.1). Because the nature of the experiment

made it meaningless to measure the protein cocentration of samples being loaded, and because there was no protein that could be used as a loading control, great care was always taken to ensure that equal volumes of supernatant were collected from each condition and that samples within an experiment were always treated identically. Cells were not counted at the time of plating but equal volumes were always added accurately to each well or plate from a constantly agitated cell suspension.

2.6 Adenovirus

2.6.1 Large-scale amplification of viral stocks

Large scale amplification of Adenovirus was carried out in HEK 293 cells according to the method outlined by He *et al.* (He et al., 1998), with modifications.

For a typical large-scale virus preparation, 20 X 150 cm^2 culture flasks of HEK 293 cells were grown to 50% confluence. 50 mL of infective supernatant was added to 150 mL of DMEM, containing 5% (v/v) fetal bovine serum. The infective supernatant was obtained by collecting the supernatant from 5X150 cm^2 culture flasks of HEK 293 cells, infected with purified virus at a concentration of 1 infectious unit (ifu) per cell.

HEK cells were incubated at 37°C for 2 h in 10 mL per flask of the diluted virus, after which 25 mL of DMEM containing 5% fetal bovine serum was added to each flask and the cells were incubated for 48 h at 37°C. After the incubation period, cells were examined for visual signs of virus infection (cell death). When all of the flasks were showing signs of virus infection, the supernatant was poured into sterile 50 mL tubes and centrifuged at 2000 x *g* for 5 min at room temperature. The resulting pellets were pooled into one sterile 50 mL tube with 25 mL sterile PBS and centrifuged at 2000 x *g* for 5 min at room temperature. The resulting pellet was re-suspended in 5 mL sterile PBS and incubated at room temperature for 10 min with 7 mL 1,1,2- trichlorotrifluoroethane. The preparation was centrifuged at 2000 x *g* for 5 min at room temperature. The top layer of the preparation, containing the virus particles was removed and place atop a CsCl step gradient (2.5 mL of 1.4 *g*/mL in 10 mM tris pH 8.1 on the bottom and 2.5 mL of 1.2 *g*/mL in 10 mM tris pH 8.1 on the top) in an SW40 centrifuge tube. The preparation was centrifuged at 92508 x *g* at 8°C for 90 min with no brake in a Beckman ultracentrifuge, using an SW40 rotor. The distinct band of virus particles appeared just below the interface and was removed by puncturing the side of the tube with a needle attached to a syringe. The virus

band was removed and dialysed overnight, at 4°C in a Pierce slide-a-lyser, with a 10K molecuar weight cut-off, against 1 L of TE pH 7.9. Following dialysis the virus was aliquoted and stored at -80°C. One aliquot of frozen virus was removed, thawed and titred.

2.6.2 Determination of Adenovirus Titer

Adenovirus titer was determined using the AdenoX™ Rapid Titer Kit from Clontech. The kit uses a hexon-specific antibody which is used to label infected cells. Each labelled cell is equivalent to one infectious unit (ifu).

2.7 TCA Precipitation of Proteins

To each 1 mL of protein solution, was added 50 µL of 0.15% (w/v) Sodium deoxycholate solution. After mixing well and incubating at room temperature for 5 min, 100 µL of 72% (w/v) TCA solution was added. Samples were mixed well, incubated on ice for 20 min, and centrifuged at 10,000 x *g* for 15 min at 4°C. The supernatant was aspirated and pellets re-suspended in 1 mL acetone (-20°C), and the centrifugation step repeated. The supernatant was aspirated and the pellets allowed to air dry for a few minutes, before being re-suspended in 60 µL 1X SDS-PAGE sample buffer (see Section 2.2.2) and stored at -80°C.

2.8 Endosome Ablation

3T3-L1 adipocytes were incubated at 37°C for 1 h in serum-free DMEM followed by 1 h in serum free DMEM containing 20 µg/mL Tf-HRP conjugate. The cells were then placed on ice, washed 3X with cold isotonic citrate buffer (150 mM NaCl, 20 mM tri-sodium citrate, pH 5.0) over a period of 10 min. 3,3'-diaminobenzidine was added at a concentration of 100 µg/mL to all plates and H_2O_2 was added at a concentration of 0.02% (v/v) to experimental plates only. After incubating for 60min at 4°C in the dark, the reaction was stopped by washing the cells with ice-cold PBS.

2.9 Subcellular fractionation of 3T3-L1 adipocytes

The LDM (Low density microsomal) fraction was obtained as described for the fractionation of rat adipocytes (Piper et al., 1991), with modifications (Martin et al., 1994).

3T3-L1 adipocytes cultured on 10 cm plates were incubated for 2 h at 37°C in serum-free DMEM. For the last 15 min of the incubation period insulin (1μM) was added to experimental plates. Plates were transferred to ice, and cells were washed 3X with 5 mL ice-cold HES buffer. Cells were then scraped in 0.5 mL per plate of HES buffer containing protease inhibitors (complete Protease Inhibitors from Roche), and homogenised with a Dounce homogeniser. The homogenate was transferred to pre-chilled centrifuge tubes and centrifuged at 16,000 x *g* for 20 min at 4°C. The resulting supernatant was centrifuged at 46,000 x *g* for 20 min at 4°C, yielding a pellet designated as the high density microsomal fraction (HDM). The resulting supernatant was centrifuged at 180,000 x *g* for 60 min at 4°C yielding a pellet containing the low density microsomal fraction (LDM). Protein was quantified using the Bradford (Bio-Rad) determination kit (section 2.9.1)

2.9.1 Bradford (Bio-rad) Protein Quantification

Five dilutions of a BSA protein standard were prepared within the linear range of 0.2-0.9 mg/mL for BSA. 20μL of each standard and sample were pipetted into a 1.5mL eppendorf. 1mL of dye reagent (prepared by diluting 1 part Dye Reagent Concentrate with 4 parts deionised water) was added to each tube and vortexed. After incubation for 5min at room temperature, absorbance was measured at 595nm, using a spectrophotometer. Sample values were plotted against a standard curve generated by the absorbance of the known dilutions of BSA.

2.10 Sucrose Gradients

Gradients were prepared as described by Guilherme *et al.* (Guilherme et al., 2000b).

Day 8 3T3-L1 adipocytes were incubated for 2h at 37°C in serum-free DMEM. For the last 15min of the incubation period, insulin (1μM) was added to experimental plates. LDM samples were prepared as described in section 2.9 Protein was quantified using the Bradford assay (Bio-Rad) (see section 2.9.1) and 1.5-2.0 mg of LDM fraction was loaded atop a 13mL 10-35% (w/v) sucrose velocity gradient in (Sucrose Gradient Buffer: 20mM HEPES pH7.4, 100mM NaCl, 1mM EDTA, protease inhibitors) and centrifuged for 3.5 h at 105,000 x *g* at 4°C in an SW40 rotor (Beckman). 1mL fractions were collected from the top to the bottom of the gradient. 20μL was removed from each fraction and immunoblots were performed to identify the GLUT4 containing fractions

The fractions containing most of the Glut4 membranes, as detected by Western blotting, were pooled, pelleted by centrifugation at 180,000 x g for 60 min at 4°C, and re-suspended in Sucrose Gradient Buffer. This resuspended pellet was then loaded on top of a 10-65% (w/v) equilibrium density sucrose gradient and centrifuged at 218,000 x g for 18 h at 4°C in an SW40 rotor. Following centrifugation, fractions were collected, starting from the top of the gradient.

2.11 2 deoxy-D-glucose uptake assay

Glucose uptake in 3T3-L1 adipocytes was measured by the uptake of [^{3}H] 2-deoxy-D-glucose (Gibbs et al., 1988), (Frost and Lane, 1985).

3T3-L1 adipocytes cultured in 12 well plates were washed 2X with 1 mL KRP buffer at 37°C. Cells were then incubated in 450 µL per well of KRP ± Insulin (1nm or100nm) at 37°C for 15 min. Duplicate control wells were incubated with the above + 10µM cytochalasin B. Glucose transport was initiated by the addition of 50 µL of [^{3}H] 2-deoxy-D-glucose (final concentration of 50µM and 0.5µ Ci/mL). After 5 min transport was terminated by flipping the plates to remove the incubation buffer and washing them by dipping the plates in three washes of ice-cold PBS. The plates were left to air dry, after which 1 mL of 1% TritonX-100 was added to each well. Plates were wrapped in film and incubated overnight at room temperature. The detergent solubilised [^{3}H] 2-deoxy-D-glucose was determined by liquid scintillation counting.

2.12 Oil Red O staining of adipocytes

3T3-L1 adipocytes were grown in 12 well culture plates. Growth medium was removed and cells were fixed in 10% formaldehyde for at least 1 h. Formaldehyde was removed and cells were washed in 60% (v/v) isopropanol and left to air dry. Cells were stained with 400 µL per well of Oil Red O solution for 10 min, after which well were rinsed with 4 changes of running dH_2O and plates were left to air dry. Oil Red O was eluted with 1.5 mL 100% isopropanol per well for 10 min. Oil Red O/isopropanol solution was transferred from each well to a 1.5 mL cuvette and OD_{520} was measured using 100% isopropanol as a blank.

2.13 SDS-PAGE

SDS-polyacrylamide gel electrophoresis was carried out using the Bio-Rad Mini-PROTEAN 3 system.

The resolving gel was prepared to the required acrylamide concentration for each experiment using 30% acrylamide/bisacrylamide solution, 375mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.01% TEMED. The stacking gel was prepared using 5% (w/v) acrylamide/bisacrylamide, 125mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1%(w/v) ammonium persulphate and 0.01% TEMED.

Protein samples were re-suspended in 1X SDS-PAGE sample buffer and loaded onto the wells in the stacking gel. Pre-stained molecular weight markers were routinely loaded in at least one well of each gel. Gels were electrophoresed at a constant voltage until sufficient separation of the molecular weight markers had been achieved.

2.14 Western Blotting

Proteins were transferred from the polyacrylamide gel to nitrocellulose membrane using the Bio-Rad Mini Trans-Blot Cell. This transfer was routinely carried out at a constant current of 90 mA, overnight.

2.15 Immunodetection of Proteins

Following transfer of proteins, the nitrocellulose membrane was incubated for 1 h in PBS containing 5%(w/v) Marvel. The membrane was then incubated with primary antibody, diluted in PBS containing 1% (w/v) Marvel washed 5X with PBS over a 30 min time period, incubated with horseradish-peroxidase labelled secondary antibody in PBS ontaining1% Marvel and washed as before.

Times and Temperatures of incubations are antibody specific.

2.15.1 Enhanced Chemiluminescent Detection

Following incubation with the appropriate HRP-linked antibody, the membrane was incubated for 1 min in a solution comprising equal volumes of the following two solutions;

Solution A 100 mM Tris-HCl pH 8.5, 2.25mM luminol, 0.4mM p-Coumaric acid, 1.44% (v/v) DMSO

Solution B 100 mM Tris-HCl pH 8.5, 0.018% (v/v) H_2O_2

Following incubation in the ECL solution, the membrane was exposed to X-Omat film and developed using an X-Omat processor.

2.15.2 Quantification of immunoblot intensities

Autoradiographs obtained by the ECL process were scanned and individual bands were densitometrically quantified using Image J, the public domain image processing programme provided by NIH (http://rsb.info.nih.gov). Unless otherwise stated the data from each blot was scanned and the values expressed as a percentage of the control. Three independent experiments were combined and error Standard deviation calculated using Excel. Where appropriate a student's T test was carried out (also using Excel). Significance was assessed as $p \leq 0.05$.

2.16 Coomassie staining of SDS-PAGE gels

For in gel detection of proteins following SDS-PAGE, gels were stained for ≥1 h at room temperature with Coomassie blue stain, after which they were destained, using Coomassie destain, until protein bands were clearly visible and background staining had been removed.

2.17 Proteomics

2.17.1 Preparation of sample for Proteomic analysis

10 cm plates of day 10 3T3-L1 adipocytes were washed 5X using 10 mL serum-free DMEM and once with serum-free DMEM (without phenol red). The cells were incubated in 8ml serum-free DMEM without phenol red for 10 h. Conditioned medium was collected, centrifuged for 10 min at 2000 x *g* at 4°C, after which the supernatant was passed through a 0.2 μm filter. The filtered supernatant was dialysed overnight, against 5 L of dH_2O in a Pierce Slide-a-Lyser dialysis cassette, with a 3.5 kDa molecular weight cut-

off, and a 3-12 mL capacity. The dialysed sample was concentrated in a vacuum centrifuge until the volume was concentrated to approximately 100 μg of protein in a volume of ≤ 100 μl..

2.17.2 1D LC-MS/MS

1D LC-MS/MS was performed in the Sir Henry Wellcome Functional Genomics Facility and the following protocol was provided by Dr Andy Pitt:

Trypsin digest was performed at 37°C overnight in 25 mM ammonium bicarbonate, 0.1% n-octyl-glycoside. Tryptic peptides were dried, resolubilized in 0.5% formic acid and fractionated by nanoflow HPLC on a C18 reverse phase column, eluting with a continuous linear gradient to 40% acetonitrile over 20 minutes. Eluate was analysed by online electrospray tandem mass spectrometry using a Qstar Pulsar XL (Applied Biosystems).

Mass spectrometric analysis was performed in IDA mode (AnalystQS software, Applied Biosystems), selecting the four most intense ions for MSMS analysis. A survey scan of 400–1500 Da was collected for 3 ses followed by 5 sec MSMS scans of 50–2000 Da using the standard rolling collision energy settings. Masses were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the Mascot (Matrix Science) search engine for protein identification.

2.17.3 Prediction of Protein Localisation

Data obtained from the MASCOT search engine was analysed for potential secreted proteins. Several prediction programmes were used during this analysis;

PSORT (Nakai and Horton, 1999) is a programme for prediciting the subcellular localisation of proteins based on their amino acid sequence. It makes predictions based on known sorting signal motifs. **WoLF PSORT** (Horton et al., 2006) is an updated version of PSORT and was also used in this study.

SignalP 3.0 Server (Bendtsen et al., 2004b) predicts traditional N-terminal signal peptides that target proteins to the ER for passage to the secretory pathway. It also recognises signal anchors (signal peptides that are not cleaved by signal peptidase and are a feature of typeII membrane proteins).

SecretomeP 2.0 Server (Bendtsen et al., 2004a) predicts non-classical protein secretion i.e. not signal peptide-triggered. Prediction is based on the observation that secreted proteins share certain features, regardless of the secretion pathway. Non-classically secreted proteins may be distinguished by properties such as amino acid composition, secondary structure and disordered regions.

Chapter 3 The mechanism of ACRP30 secretion in adipocytes

3.1 Introduction

ACRP30 is one of the most abundant proteins to be secreted by adipocytes but in common with many of the other adipokines there is little known about the pathway it follows between its synthesis in the cytosol and its eventual secretion to the extracellular space. Insulin increases the secretion of a range of molecules from adipocytes including adipsin and ACRP30. Adipsin and GLUT4 are known to traffic through the TGN and their increased secretion is due in part to an increase in vesicle transport at the TGN. Adipsin has however been shown to traffic from the TGN to the PM via endosomes, as its secretion is inhibited by endosome ablation and by treatment of the cells with the PI-3 kinase inhibitor Wortmannin (Millar et al., 2000), whereas GLUT4 is still transported to the cell surface following endosome ablation. GLUT4 is found in basal cells to cycle between a population of intracellular vesicles that are positive for the v-SNARE VAMP2 (Ramm et al., 2000) and the plasma membrane. Upon insulin stimulation, there is a rapid shift in localisation from these VAMP2 positive secretory vesicles to the PM. As well as these insulin-responsive storage vesicles, GLUT4 colocalises with the TGN and the endosomal recycling compartment (Bryant et al., 2002) and is proposed to involve two main intracellular recycling pathways: pathway 1 cycling between the PM and endosomes and pathway 2 between the TGN and endosomes Figure 3.1 (Bryant et al., 2002).

In this study I have addressed questions regarding the mechanisms of secretion of ACRP30, namely how does ACRP30 traffic to the PM? Is it transported directly from the TGN to the PM in a constitutive manner, or is it packaged into immature storage granules and trafficked to the cell surface in a regulated fashion like GLUT4 ? Alternatively, does ACRP30, like adipsin, traffic to the cell surface via endosomes? In addressing this question I have employed a technique called endosome ablation (Livingstone et al., 1996) in which a Tf-HRP conjugate is taken up by 3T3-L1 adipocytes and delivered to the TfR positive endosomal compartment. Subsequent incubation of the cells with DAB and hydrogen peroxide results in cross-linking of the TfRs, and loss of immunoreactive TfR from intracellular membranes. This procedure ablates markers of the recycling endosome including TfR and Rab5 with little or no effect on markers of the TGN (Livingstone et al., 1996; Martin et al., 1996).

Many proteins trafficking to the PM, including GLUT4, recycling transferrin receptors and adipsin use the endocytic pathway (Chakrabarti et al., 1994; Ang et al., 2004; Millar et al., 2000) . ACRP30 and GLUT4 have been located by convolution immunofluorescence

microscopy to distinct subcellular compartments (Bogan and Lodish, 1999) but depletion of the the v-SNARE Vti1a decreases both insulin stimulated deoxyglucose uptake and ACRP30 secretion suggesting the existence of an element in the secretory pathway that is common to GLUT4 and ACRP30. Despite these findings, little is known about the trafficking of ACRP30.

The exocyst is a complex of eight protein subunits that are involved in the docking of secretory vesicles at the PM (Figure 1.4). Although it was first identified in yeast, it is well conserved with mammalian homologues having been identified for all eight proteins (Hsu et al., 1996). Several of the subunits of the exocyst have been identified as effectors of GTPases. Sec4, a member of the Rab family of small GTPases, interacts with Sec 15 (Guo et al., 1999) and Rho1, Rho3, Cdc42 and Ral1 all interact with exocyst proteins (Novick and Guo, 2002; Zhang et al., 2001). In mammalian cells the exocyst, or Sec6/8 complex, co-localises with exocytic cargo protein on the TGN and at the PM and is necessary for transport between these two membranes (Yeaman et al., 2001). The exocyst has been reported to regulate the sorting of GLUT4 into lipid rafts in adipocytes (Inoue et al., 2006) and is proposed to function in regulated exocytosis (Yeaman et al., 2001).

I have also examined the roles of the syntaxins 6, 8 and 12 in the regulation of ACRP30 secretion. Syntaxins are t-SNARES that are involved in vesicle docking and fusion in the secretory and endocytic pathways. To date 18 syntaxins have been identified and they are localised to different intracellular compartments(Pelham, 2001). Syntaxins 1-4 are localised to the plasma membrane and Syntaxins 6,7, 8 and 12 are involved in the TGN and endosomal network (Watson and Pessin, 2000; Tang et al., 1998).

3.2 Experimental Procedures

3.2.1 ACRP30/Adipsin Secretion Assay

Mature 3T3-L1 adipocytes were grown in either 10cm plates or 12-well plates. At days 8-11 post-differentiation, cells were serum-starved for 2h in serum-free DMEM at 37°C. Where the procedure was carried out in 10cm plates, cells were washed three times with 10mL Krebs Ringer Phosphate buffer (KRP) and at time t=0 this was replaced with 5mL fresh KRP± 1μM Insulin at 37°C. At each time point, 0.5mL KRP was removed to an eppendorf. In the case of 12 well plates KRP was replaced with 0.5mL fresh KRP± 1μM Insulin at 37°C, and at various time points, indicated in each experiment, this was removed

to an eppendorf. In the 12 well plates, each condition was performed in three neighbouring wells, and each experiment was performed three times.

In both cases the 0.5mL sample was TCA precipitated (section 2.7), the pellet was resuspended in 50μL SDS-PAGE sample buffer containing 20mM DTT (section 2.2.2) and 20μL was loaded onto a 10% SDS-PAGE gel (section 2.13), and electro blotted onto nitrocellulose. Western blots were blocked with 5% Marvel in PBS for 1h at room temperature, followed by incubation with either rabbit anti-ACRP30 anti-serum diluted 1:2000 or goat anti-adipsin anti-serum diluted 1:1000 in PBS containing 1% Marvel for 1h at room temperature. After thorough washing with several changes of PBS, blots were incubated with Horseradish Peroxidase (HRP)-conjugated donkey anti-rabbit IgG (whole molecule) or HRP-conjugated rabbit anti-goat IgG diluted 1:1000 in PBS containing 1% Marvel for 1h at room temperature. Following thorough washing with several changes of PBS, the antigen-antibody complex was detected with enhanced chemiluminescence (see section 2.15.1) and visualised by autoradiography. The autoradiographs were scanned and quantified densitometrically, using the image-processing programme Image J. The value obtained for each was expressed as a percentage of the value for the control band in each experiment, and the results shown are the combination of three experiments (unless stated otherwise).

3.2.2 Purification of Insulin-responsive GLUT4-containing membranes

Day 8 3T3-L1 adipocytes were incubated for 2h at 37°C in serum-free DMEM. For the last 15min of the incubation period, insulin (1μM) was added to experimental plates. LDM samples were prepared as described in section 2.9 Protein was quantified using the Bradford assay (Bio-Rad) (see section 2.9.1). 1.5-2.0 mg of LDM fraction were loaded atop a 13mL 10-35% (w/v) sucrose velocity gradient in (Sucrose Gradient Buffer: 20mM HEPES pH7.4, 100mM NaCl, 1mM EDTA, containing protease inhibitors) and centrifuged for 3.5 h at 105,000 x *g* at 4°C in an SW40 rotor (Beckman). 1mL fractions were collected from the top to the bottom of the gradient. 20μL was removed from each fraction and immunoblots were performed to identify the GLUT4 containing fractions.

The fractions containing most of the Glut4 membranes (fractions 6-10), as detected by Western blotting were pooled, pelleted by centrifugation at 180,000 x g for 60 min at 4°C, and re-suspended in Sucrose Gradient Buffer. This resuspended pellet was then loaded atop a 10-65% (w/v) equilibrium density sucrose gradient and centrifuged at 218,000 x *g* for 18 h at 4°C in an SW40 rotor. Following centrifugation, fractions were collected,

starting from the top of the gradient. 15μL of each fraction was analysed on a 10% SDS-PAGE gel.

3.2.3 Ablation of Transferrin receptor-positive endosomal compartment

Four 10cm plates of day 8-11 mature 3T3-L1 adipocytes were incubated at 37°C for 1 h in 10mL serum-free DMEM followed by 1 h in 5mL serum free DMEM containing 20 μg/mL Tfn-HRP conjugate. The cells were then placed on ice, washed 3X with cold isotonic citrate buffer (150 mM NaCl, 20 mM tri-sodium citrate, pH 5.0) over a period of 10 min. 3,3'-diaminobenzidine was added at a concentration of 100 μg/mL to all plates and H_2O_2 was added at a concentration of 0.02% (v/v) to experimental plates only (2 plates). After incubating for 60min at 4°C in the dark, the reaction was stopped by washing the cells with ice-cold PBS. Cells were then washed 3X with KRP at 37°C, before being assayed for secretion of ACRP30 and adipsin.

3.2.4 Infection of 3T3-L1 adipocytes with adenoviruses driving the overexpression of Sec6, Sec8, Syntaxin6, Syntaxin8 and Syntaxin12

Day 8 mature 3T3-L1 adipocytes, grown in 12-well culture plates were infected with adenovirus at an moi of 50pfu/cell. The virus was added to the cells in 0.5mL serum-free DMEM for 4h, after which time 0.5mL DMEM containing 10% (v/v) FCS was added to each well. Cells were incubated with virus for 48h, before being assayed for secretion of ACRP30.

3.2.5 Brefeldin A treatment of 3T3-L1 adipocytes

3T3-L1 adipocytes, grown in 10 cm culture dishes, were incubated with 5 μg/mL of Brefeldin A at 37°C for 1 h before assaying for secretion of ACRP30.

3.3 Results

3.3.1 Characterisation and affinity purification of anti-ACRP30 antibody

An antibody was raised in rabbits against a peptide corresponding to the C- terminal 18 amino acid residues (226-244) of the mouse ACRP30 protein, and is shown underlined in the full sequence below.

MLLLGAVLLLLALPGHDQETTTQGPGVLLPLPKGACTGWMAGIPGHPGHNGAPG
RDGRDGTPGEKGEKGDPGLIGPKGDIGETGVPGAEGPRGFPGIQGRKGEPGEGAY
VYRSAFSVGLETYVTIPNMPIRFTKIFYNQQNHYDGSTGKFHCNIPGLYYFAYHITV
YMKDVKVSLFKKDKAMLFTYDQYQENNVDQASGSVLLHLEVGDQVWLQVYGE
GERNGLYADNDNDSTFTGFLLYHDTN

This was used to study secretion of ACRP30 from 3T3-L1 adipocytes. Figure 3.2a shows the secretion of a protein of 30kDa from adipocytes over 60 min. This illustrates the constitutive secretion of ACRP30. Figure 3.2b shows secreted supernatant from basal and insulin stimulated 3T3-L1 adipocytes blotted with pre-immune serum from the immunised rabbit.

3.3.2 Secretion of ACRP30 is adipocyte specific

It is known that differentiation of 3T3-L1 adipocytes results in the activation of transcription factors and the subsequent expression of adipocyte specific proteins. ACRP30 is one of the proteins that are abundantly secreted by 3T3-L1 adipocytes. In order to investigate whether ACRP30 was secreted by 3T3-L1 fibroblasts as well as differentiated adipocytes, 3T3-L1 pre-adipocytes and 3T3-L1 adipocytes were incubated for 2 h in serum-free DMEM before being assayed for secretion of ACRP30 as described in Section 2.5.

Figure 3.3 illustrates that ACRP30 could not be detected in the supernatant of undifferentiated 3T3-L1 fibroblasts, confirming that it is not expressed in 3T3-L1 fibroblasts.

3.3.3 Constitutive and insulin-stimulated secretion of ACRP30 from 3T3-L1 adipocytes

3T3-L1 adipocytes were cultured in 10 cm plates and incubated in serum-free DMEM before being assayed for secretion of ACRP30. Cells were incubated at 37°C in KRP ±

1μM Insulin, and samples were collected at defined time points between 2 min and 30 min. Samples were assayed for ACRP30 secretion as described in Section 2.5 and in more detail in the experimental procedures section in this chapter (section 3.2.1).

Figure 3.2a shows that ACRP30 is constitutively secreted constitutively from 3T3-L1 adipocytes. Figure 3.4 illustrates that insulin increases the rate of secretion over a short time, with the rate of secretion paralleling basal rates within 30 min-1 h.

3.3.4 Intracellular distribution of ACRP30 in adipocytes

Crude subcellular fractionation experiments have previously shown that secretory proteins such as ACRP30 and adipsin, localise mainly to low density microsomal fractions, in common with GLUT4 (Millar et al., 2000). To more accurately determine the subcellular distribution of ACRP30, I compared the localisation of ACRP30 with that of GLUT4/ IRAP (the Insulin-responsive aminopeptidase) and the endosomal marker TfR by resolving intracellular membranes on velocity (Figure 3.5a) and equilibrium density gradients (Figure 3.5b). This two step gradient analysis results in an enrichment of insulin responsive GLUT4-containing vesicles (Guilherme et al., 2000a). After the velocity gradient, (Figure 3.5a) the majority of ACRP30 was localised to the same fractions as those containing Glut4/IRAP or transferrin receptors (TfR). The ACRP30-rich fractions (3 to 9 of Fig 3.5a) were collected, pooled and subjected to density gradient analysis. This was repeated for the insulin-stimulated fractions. Fig 3.5b shows fractions 4 to 9 of the basal and insulin-stimulated fractions. This analysis confirms the previous report that this method can effectively resolve the GLUT4/IRAP-containing vesicles and shows that the GLUT4 within these vesicles is mobilised in response to insulin. ACRP30 exhibits limited overlap with GLUT4 using this method, but a greater degree of overlap with the endosomal marker (TfR). This data provides evidence for a distinct localisation of ACRP30 and GLUT4 and the overlapping localisation of ACRP30 and TfR suggests that ACRP30 is secreted in a constitutive-like manner, through the endosomal system.

3.3.5 Effect of Brefeldin A on secretion of ACRP 30

Brefeldin A inhibits protein secretion and intracellular protein transport by preventing Arf 1 recruitment of COPI coat protein to Golgi membranes (Orci et al., 1991; Misumi et al., 1986). In order to investigate the involvement of Golgi trafficking in ACRP30 secretion I treated 3T3-L1 adipocytes with Brefeldin A, before assaying for ACRP30 secretion.

3T3-L1 adipocytes were incubated in DMEM-SF for 1 h followed by DMEM-SF containing 5 μg/mL Brefeldin A for 1 h before assaying for the secretion of ACRP30 as described in Section 2.5. Fig 3.6 illustrates that BFA treatment strongly inhibits both basal and insulin stimulated secretion of ACRP30, consistent with trafficking of these proteins through the TGN.

3.3.6 Effect of endosome ablation on ACRP30 secretion.

It has been shown previously that disruption of the TfR positive endosomal compartment is inhibitory for adipsin secretion. In order to determine the involvement of the endosomal system during trafficking of ACRP30, I disrupted the transferrin receptor positive endosomal system by loading the cells with TfHRP and incubating with hydrogen peroxide. After this procedure, the cells were assayed for secretion of ACRP30. Fig 3.7 shows that disruption of this compartment strongly inhibits ACRP30 secretion, suggesting that during secretion, ACRP30 and adipsin follow a pathway that involves the recycling endosomal compartment. This is consistent with the observation that a fraction of the intracellular ACRP30 co-localises with TfR positive membranes (Figure 3.5b)

3.3.7 Role of the exocyst proteins Sec6 and Sec8 in ACRP30 secretion

In order to investigate the role of the exocyst proteins in ACRP30 secretion, I overexpressed the exocyst proteins Sec6 and Sec8 in 3T3-L1 adipocytes and examined the effect of this overexpression on ACRP30 secretion.

3T3-L1 adipocytes grown in 12 well culture plates and infected with with empty adenovirus (Sh) or adenoviruses designed to drive over expression of the exocyst proteins Sec6 and Sec8. The cells were infected with adenoviruses expressing full length Sec6F, Sec8F or a truncated form of each (Sec6T and Sec8T). Fig 3.8 shows a typical immunoblot of secretion of ACRP30 from these 3T3-L1 adipocytes. Over expressing Sec6 and Sec8 appears to have no effect on secretion of ACRP30. A study by Ewart *et al.* showed that adenoviral overexpression of Sec6 and Sec8 significantly enhanced the maximum rate of insulin-stimulated deoxyglucose uptake (Figure3.9)*, in agreement with the proposition that the exocyst has a role in regulated exocytosis. The results in Figure 3.8 show no evidence of a role for the exocyst in ACRP30 secretion and suggest that ACRP30 and GLUT4 traffic to the PM by distinct pathways.

*Figure 3.9 is not part of this study but is the work of M.A.Ewart (Ewart et al., 2005) and was performed contemporaneously with the data of Figure 3.8.

3.3.8 Role of Syntaxins in ACRP30 secretion

3T3-L1 adipocytes were infected with adenovirus expressing syntaxins 6, 8 or 12 and the cells were assayed for secretion of ACRP30 as described in Section 2.5.

A previous study has shown that in adipocytes ~ 85% of Syntaxin 6 is found localised with GLUT4-containing vesicles (Perera et al., 2003), and that overexpressing the cytosolic domain of Syntaxins 6, 8 and 12, using an adenoviral vector, had no effect on the maximal rate of insulin-stimulated deoxyglucose uptake. The rate of deoxyglucose transport in the absence of insulin was increased by overexpression of Syntaxin 6, implicating a role for Syntaxin 6 in the trafficking of Glut4 within the endosomal system of adipocytes. Figure 3.10 shows that the rate of decline of insulin-stimulated deoxyglucose transport after insulin withdrawal is slower in cells overexpressing Syntaxin 6, suggesting that perturbation of the Syntaxin 6 function results in GLUT4 remaining in the recycling endosomes for longer. This data suggests a role for Syntaxin6 in to GLUT4 trafficking between the endosomal compartment and GLUT4 storage vesicles.

In this study I investigated the role of the Syntaxins 6, 8 and 12 in ACRP30 secretion by overexpressing the proteins, using an adenoviral vector, in 3T3-L1 adipocytes, before assaying for secretion of ACRP30. 3T3-L1 adipocytes were infected with adenovirus designed to overexpress the cytosolic domains of Syntaxins 6, 8 and 12 and the cells were assayed for secretion of ACRP30 as described in Section 2.5. Fig 3.11 shows that the over expression of syntaxins 6, 8 and 12 has no effect on either basal or insulin stimulated secretion of ACRP30.

*Figure 3.10 is not part of this study but is the work of H.K.I. Perera (Perera et al., 2003) and was performed contemporaneously with the data in Figure 3.11.

3.4 Discussion

In this study, I have shown that, in common with adipsin, ACRP30 is secreted in a 'constitutive-like' manner by 3T3-L1 adipocytes and that although the rate of secretion is increased over a short time period in response to insulin (Figure 3.4), there is no evidence that ACRP30 exhibits regulated secretion from 3T3-L1 adipocytes. Both basal and insulin

stimulated secretion of ACRP30 are inhibited following endosome ablation (Figure 3.7), presenting further evidence that the increase in secretion seen in response to insulin (Figure 3.4) is less likely to be brought about by regulated secretion, than insulin stimulation of the recycling endosomal system.

In intracellular localisation studies, ACRP30 was found in the same fractions as GLUT4 (an example of a protein that is transported to the PM from an intracellular compartment in a regulated fashion) but showed a greater degree of overlap with the TfR positive compartment, suggesting that these two proteins may populate the same compartment. Brefeldin A treatment of 3T3-L1 adipocytes strongly inhibited basal and insulin-stimulated secretion of ACRP30, indicating that secretion of this protein is dependent on a functioning TGN. This parallels the results seen with adipsin. These data suggest that ACRP30-containing secretory vesicles traffic through the TGN and the endosomal system on their way to the plasma membrane, pointing to a constitutive-like secretory route. It has been suggested that ACRP30 is secreted via a regulated secretory pathway (Bogan and Lodish, 1999), based on a lack of co-localisation of ACRP30 with the TfR by immunofluorescence. I have however shown that in subcellular fractionation studies ACRP30 does in fact, overlap with TfR positive fractions (Figure 3.5), further evidence for a constitutive-like secretory pathway for ACRP30. These same subcellular fractionations demonstrated little overlap between ACRP30 and GLUT4-containing membranes suggesting that ACRP30 does not traffic to the cell surface using the same regulated secretory route as GLUT4.

Figure 3.8 shows that overexpressing the exocyst proteins Sec6 and Sec8 in 3T3-L1 adipocytes has no effect on secretion of ACRP30. Since the exocyst is proposed to play a role in regulated exocytosis, and there is evidence for involvement of the exocyst in GLUT4 vesicle docking at the PM, the lack of involvement of the exocyst in secretion of ACRP30 provides evidence that ACRP30 and GLUT4 traffic to the PM by distinct pathways.

Further evidence that ACRP30 and GLUT4 traffic to the PM through distinct pathways is shown in Figure 3.11. This data shows that overexpressing the Syntaxins 6, 8 and 12 has no effect on ACRP30 secretion. A previous study of the involvement of these three Syntaxins in GLUT4 trafficking to the PM suggests a role for Syntaxin6 in GLUT4 trafficking (Perera et al., 2003).

In addition, other studies have implicated Syntaxins 8 and 12 in trafficking within the endosomal system (see section3.1). I have suggested that endosomal traffic is involved in ACRP30 secretion based upon ablation and co-localisation of TfR, and therefore sought to determine whether Syntaxin 8 or 12 perturbed ACRP30 traffic. I was however unable to determine any effect, which could indicate that Syntaxins 8 and 12 are not necessary for ACRP30 traffic or may reflect an inability of the overexpressed protein to function as an inhibitor of the endogenous molecule. Since there are no available assays for the function of Syntaxin 8 and 12, this area was not pursued.

Figure 3.1 GLUT4 Trafficking

A model that depicts the transport of GLUT4 in insulin-responsive cells. The model depicts two main intracellular-recycling pathways: cycle 1, between the cell surface and endosomes; and cycle 2, between the trans-Golgi network (TGN) and endosomes. GLUT4 transport is intricately controlled at several points along these cycles. On entry into the endosomal system, GLUT4 is selectively retained at the expense of other recycling transport, such as the transferrin receptor that constitutively moves through cycle 1. This retention mechanism might predispose GLUT4 for sorting into transport vesicles that bud slowly from the endosome and that are targeted to the TGN. GLUT4 is sorted into a secretory pathway in the TGN. This sorting step probably involves a specialized population of secretory vesicles that excludes other secretory cargo, and that does not fuse constitutively with the plasma membrane. Vesicles that emerge from this sorting step, which we have previously referred to as GLUT4 storage vesicles or GSVs, might constitute most of the GLUT4 that is excluded from the endosomal system. In the absence of insulin, GSVs might slowly fuse with endosomes, thereby accounting for the presence of a significant but small pool of GLUT4 in endosomes, even in the absence of insulin. Insulin would then shift GLUT4 from this TGN–endosome cycle to a pathway that takes GLUT4 directly to the cell surface. (Bryant et al., 2002)

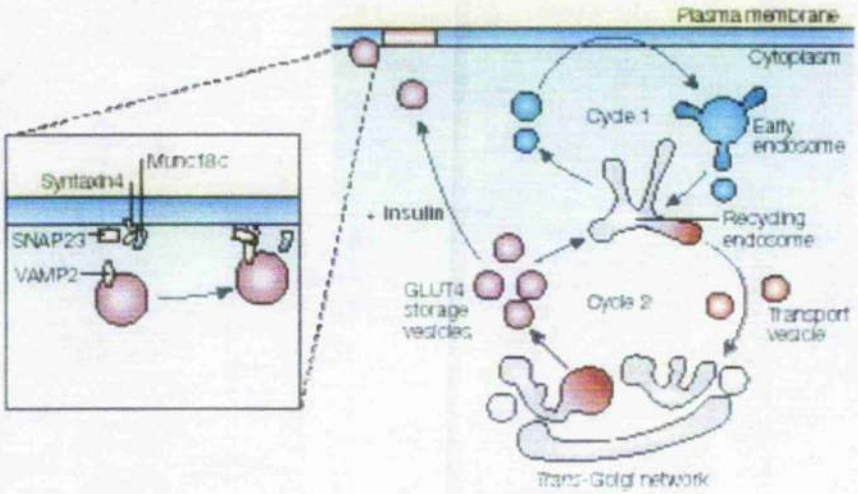

Plasma membrane
Cytoplasm
Cycle 1
Early endosome
Munc18c
Syntaxin4
SNAP23
VAMP2
Insulin
Recycling endosome
GLUT4 storage vesicles
Cycle 2
Transport vesicle
Trans-Golgi network

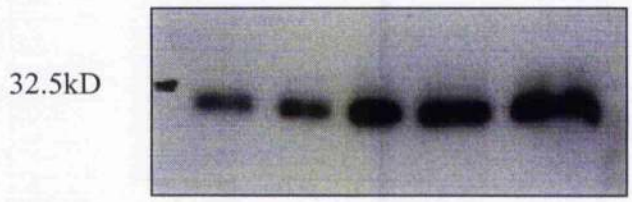
Figure 3.2 Secretion of ACRP30 by 3T3-L1 adipocytes

3T3-L1 adipocytes were assayed for secretion of ACRP30 as described in Section 2.5.

Figure 3.2a shows the secretion of ACRP30 at defined timepoints.

Figure 3.2b shows secreted proteins from 3T3-L1 adipocytes, blotted with pre-immune serum from the rabbit that was immunised during the production of the anti-ACRP30 antibody.

a

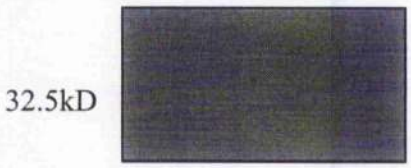


ACRP30

0 5 10 35 60 time(min)

b

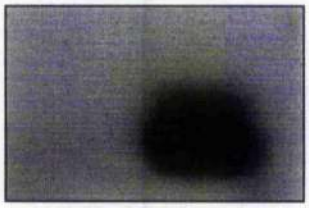
insulin - +



p.i.s

Figure 3.3 ACRP30 secretion is adipocyte specific

Pre-adipocytes and mature adipocytes were assayed for secretion of ACRP30. Only mature 3T3-L1 adipocytes secrete ACRP30



ACRP30

3T3-L1 adipocytes

3T3-L1 pre-adipocytes

Figure 3.4 Insulin-stimulated ACRP30 secretion

3T3-L1 adipocytes were grown in 12 well culture plates and assayed for secretion of ACRP30 at defined time points between 2 min and 30 min. Cells treated with insulin showed a slight increase in the rate of secretion over a short time period. Blots from two independent experiments were measured by densitometry and combined. The results shown are ± S.D.

The x-axis is non-parametric because the results show the time points collected in the experiment.

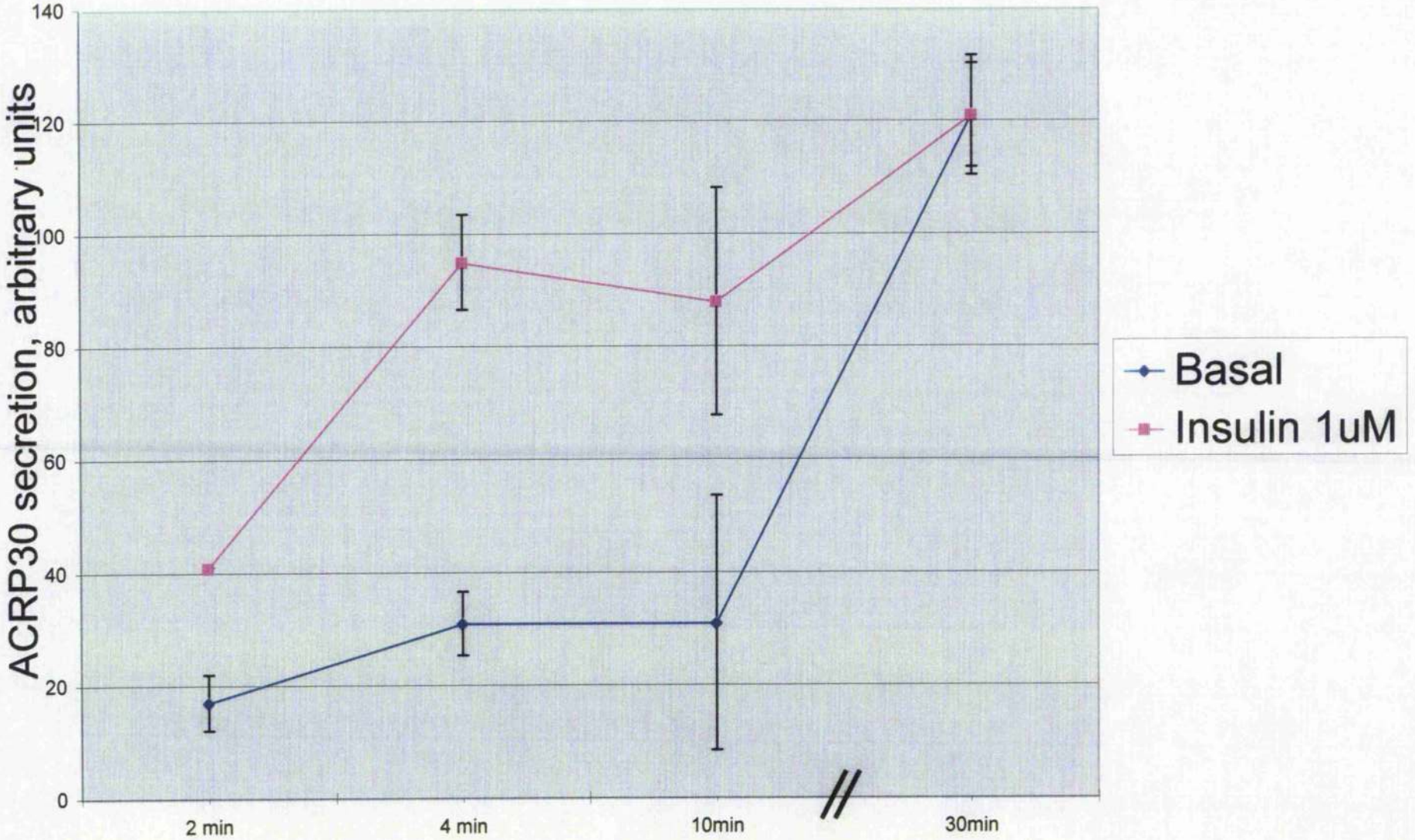
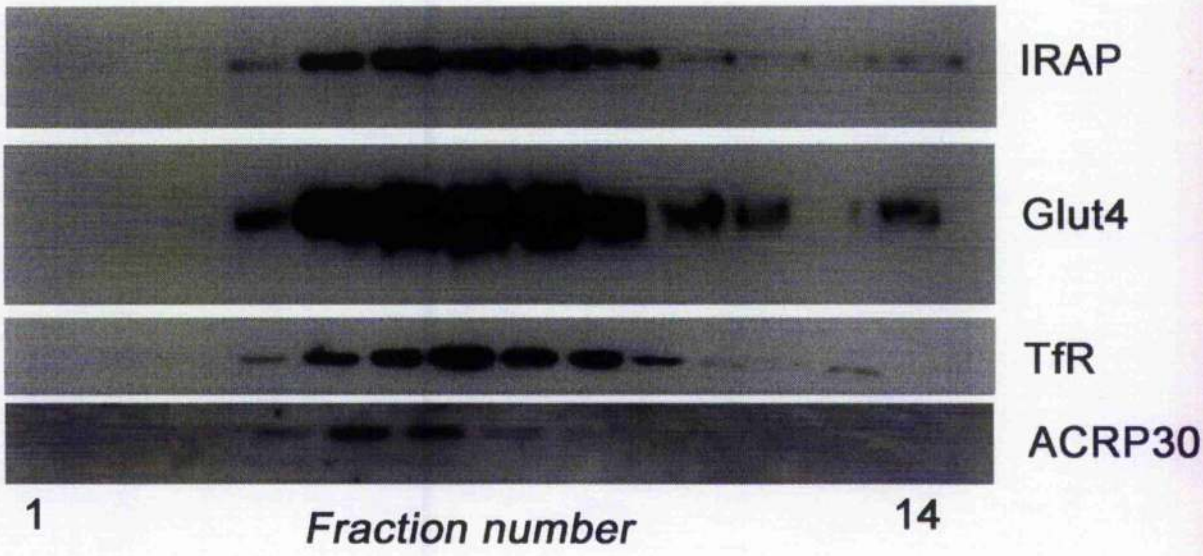

ACRP30 secretion, arbitrary units
140
120
100
80
60
40
20
0
2 min
4 min
10min
30min
Basal
Insulin 1uM

Figure 3.5 Intracellular localisation of ACRP30

3.5a. The immunoblot shown is from basal cells; insulin stimulation of 1μM for 30min did not alter the distribution among the fractions.

3.5b Shows equilibrium gradient analysisof fractions 3 through 9 of the velocity gradient. Equal amounts of the fractions were separated by SDS-PAGE and immunoblotted with tha antibodies indicated. This analysis clearly shows that ACRP30 exhibits limited overlap with Glut4, however there is more overlap of ACRP30 with the endosomal marker (TfR).

a


IRAP
Glut4
TfR
ACRP30
1
Fraction number
14

b

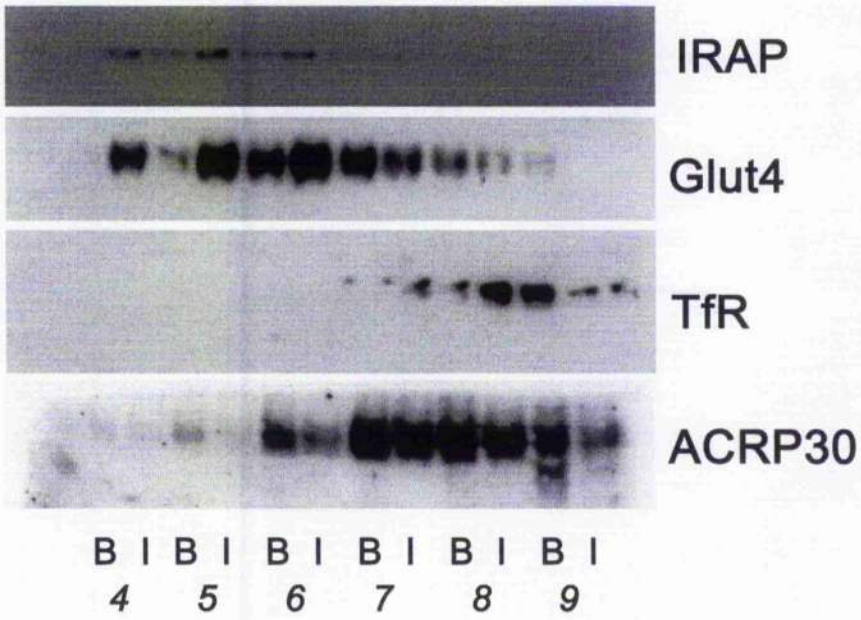

IRAP
Glut4
TfR
ACRP30
B I B I B I B I B I B I
4 5 6 7 8 9

Figure 3.6 Effect of Brefeldin A treatment on secretion of ACRP30

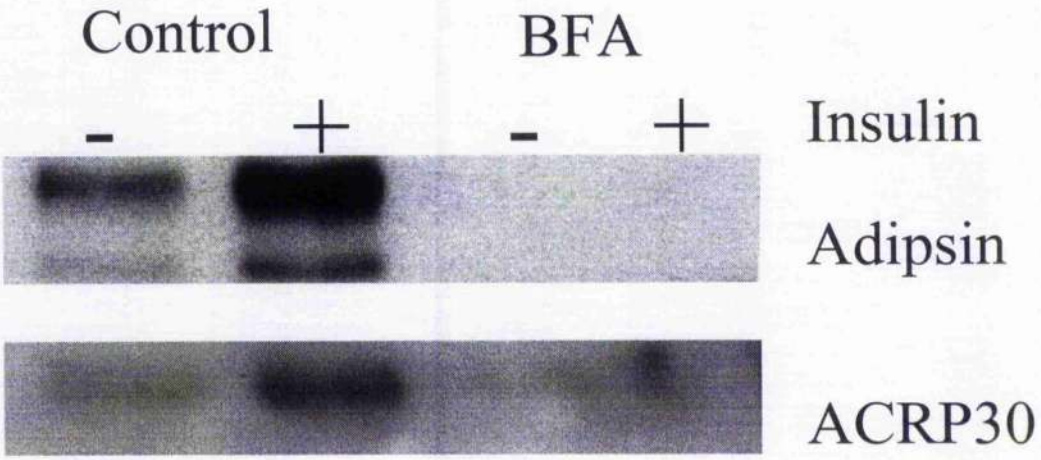
Shown in 3.6a is a representative Western blot. The blot shows that, consistent with its effect on adipsin secretion, Brefeldin A strongly inhibits secretion of basal and insulin stimulated ACRP30 secretion.

The blots from three independent experiments were scanned and the results combined.

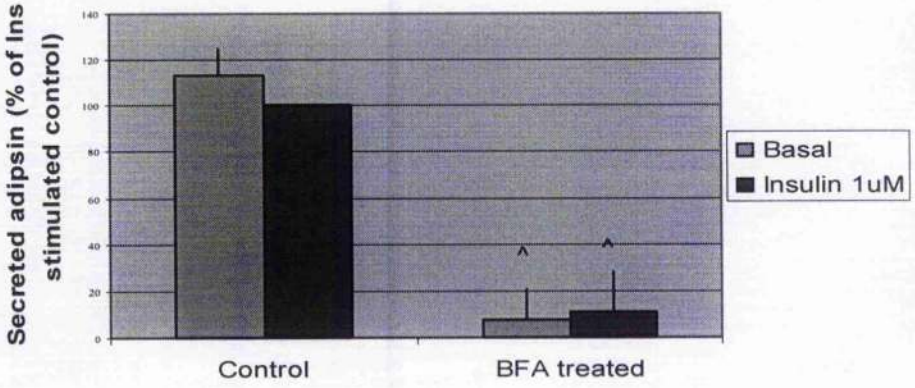
3.6b shows the results from the denstometric scans of the adipsin blots ± S.D. ^ indicates a result with value of $p \leq 0.01$.

3.6c shows the results from the denstometric scans of the ACRP30 blots ± S.D. ^ indicates a result with value of $p \leq 0.01$.

a


Control
BFA
-
+
-
+
Insulin
Adipsin
ACRP30

b


Secreted adipsin (% of Ins stimulated control)
140
120
100
80
60
40
20
0
Basal
Insulin 1uM
Control
BFA treated

c

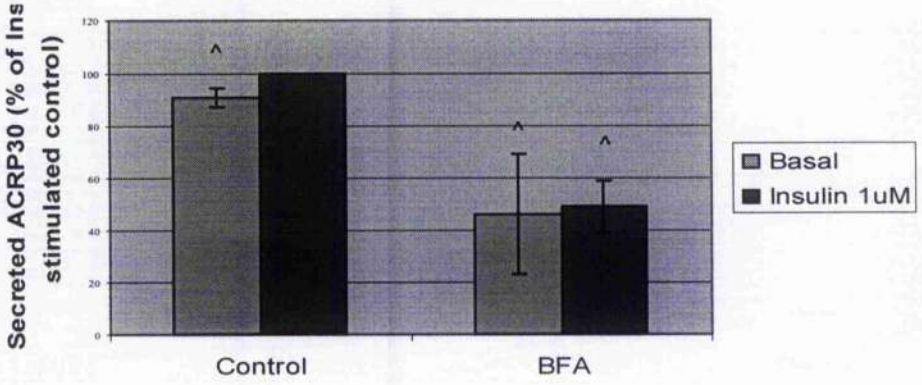

Secreted ACRP30 (% of Ins stimulated control)
120
100
80
60
40
20
0
Basal
Insulin 1uM
Control
BFA

Figure 3.7 Effect of Endosomal Ablation on ACRP30 secretion in 3T3-L1-adipocytes

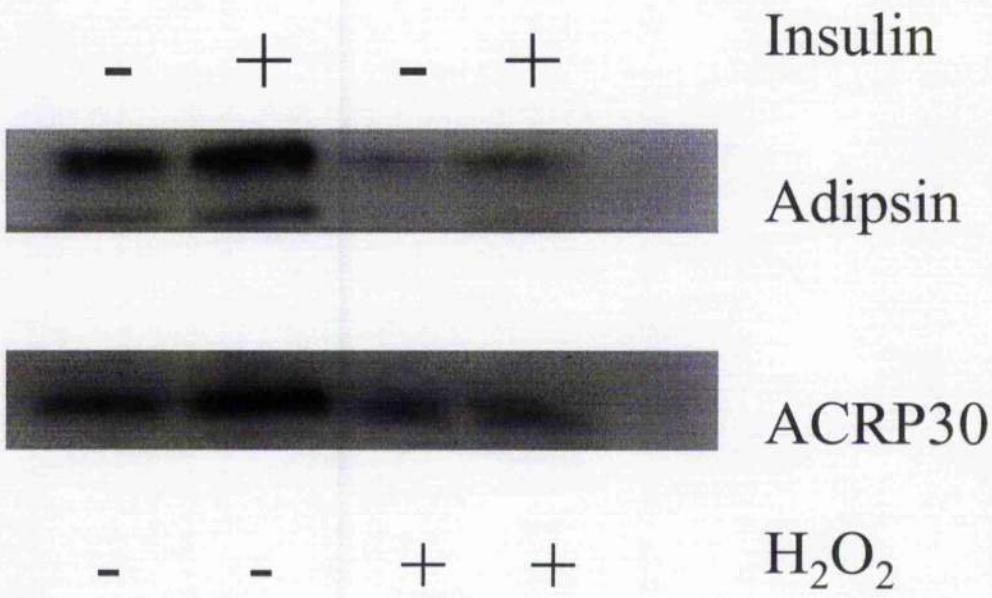
Shown in 3.7a is a representitive Western blot. The blot shows that, consistent with its effect on adipsin secretion, endosome ablation inhibits secretion of basal and insulin stimulated ACRP30 secretion.

The blots from three independent experiments were scanned and the results combined

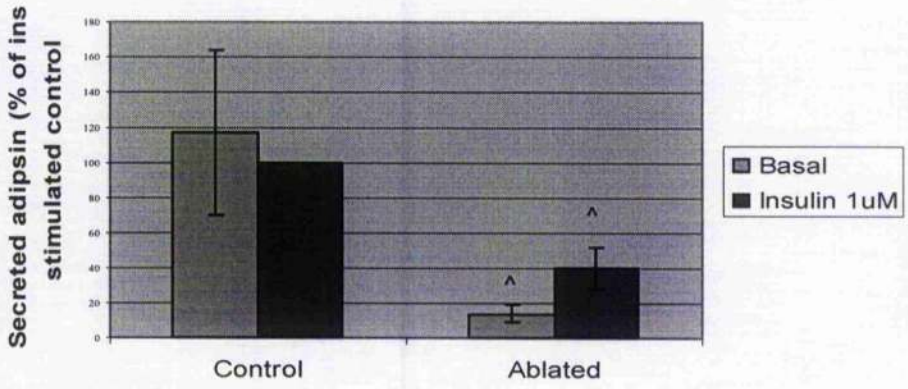
3.7 b shows the results from the denstometric scans of the adipsin blots ± S.D. ^ indicates a result with value of $p \leq 0.01$.

3.7c shows the results from the denstometric scans of the ACRP30 blots ± S.D. ^ indicates a result with value of $p \leq 0.01$.

a


- + - +
Insulin
Adipsin
ACRP30
- - + +
H_2O_2

b


Secreted adipsin (% of ins stimulated control
Control
Ablated
Basal
Insulin 1uM

c

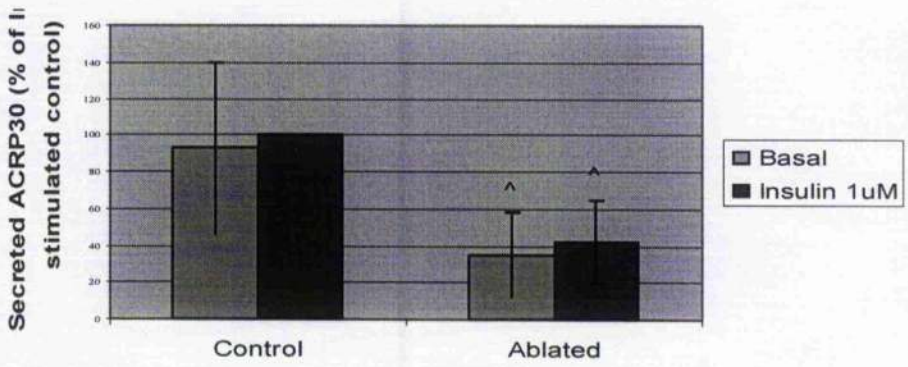

Secreted ACRP30 (% of I stimulated control)
Control
Ablated
Basal
Insulin 1uM

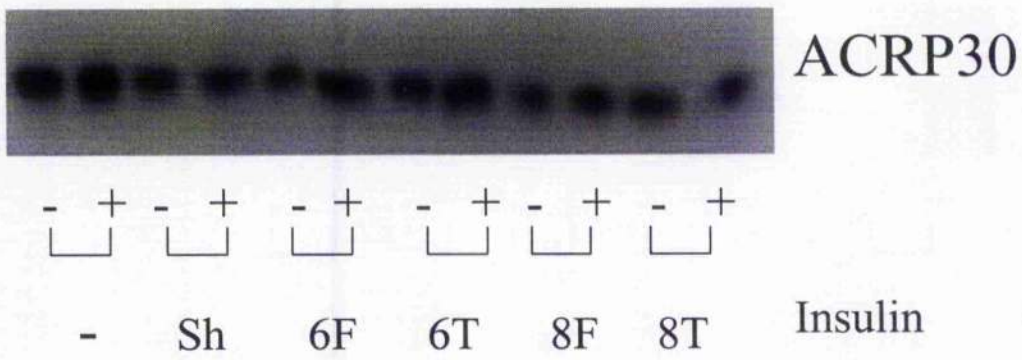
Figure 3.8 Effect of overexpression of the exocyst proteins Sec6 and Sec8 on secretion of ACRP30

Fig 3.8a shows a typical immunoblot of secretion of ACRP30 from these 3T3-L1 adipocytes.

The blots from three independent experiments were scanned and the results combined

3.8b shows the results from the densitometric scans of the ACRP30 blots ± S.D.

a


ACRP30
- + - + - + - + - + - +
- Sh 6F 6T 8F 8T
Insulin

b

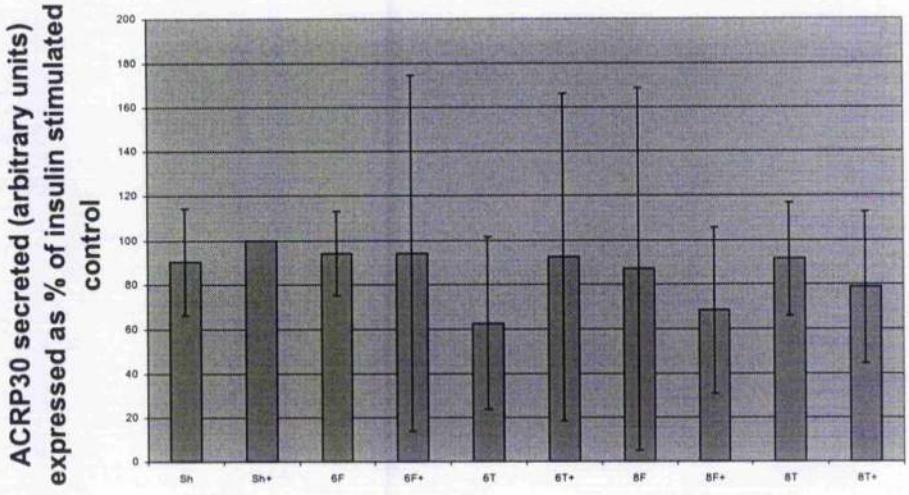

ACRP30 secreted (arbitrary units) expressed as % of insulin stimulated control
200
180
160
140
120
100
80
60
40
20
0
Sh
Sh+
6F
6F+
6T
6T+
8F
8F+
8T
8T+

Figure 3.9
Sec6 and Sec8 overexpression affect insulin-stimulated deGlc uptake

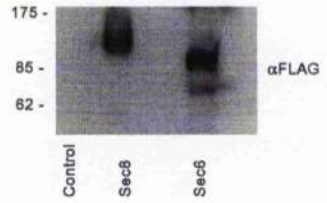
3.9a Over-expression of FLAG-tagged rsec6 or rsec8.

3T3-L1 adipocytes were infected with empty virus (control) or viruses designed to drive over-expression of rsec6 or rsec8 as described in *Materials and Methods*. Lysates were then prepared as outlined and immunoblotted with anti-FLAG antibodies. Data from a representative experiment is shown in the corner of Figure 3.9b; Figures to the left indicate the approximate positions of molecular weight markers (kDa).

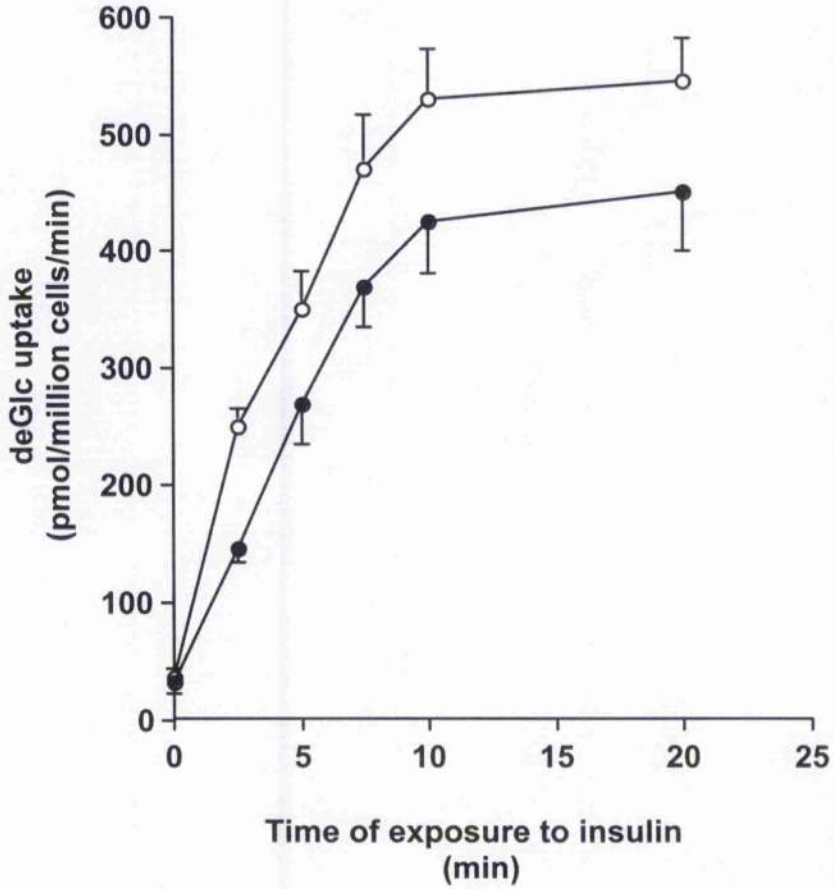
rsec6 and rsec8 over-expression increases maximal insulin-stimulated deGlc uptake.

3T3-L1 adipocytes were infected with empty virus, rsec6 or rsec8-virus and deGlc uptake determined as outlined in *Materials and Methods*. After 2h in serum-free media, cells were rapidly washed in KRP buffer at 37C, then incubated in the same in the presence or absence of insulin for the indicated times. Shown are representative experiments from over 6 of this type, each point representing triplicate determinations (± s.d.) at each condition. In Figure 3.9b, filled circles are empty-virus infected cells, open circles are cells over-expressing rsec8. In Figure 3.9c, filled circles are empty-virus infected cells, open circles are cells over-expressing rsec6.

a


175 -
85 -
62 -
αFLAG
Control
Sec8
Sec6

b


600
500
400
300
200
100
0
deGlc uptake
(pmol/million cells/min)
0
5
10
15
20
25
Time of exposure to insulin
(min)

C

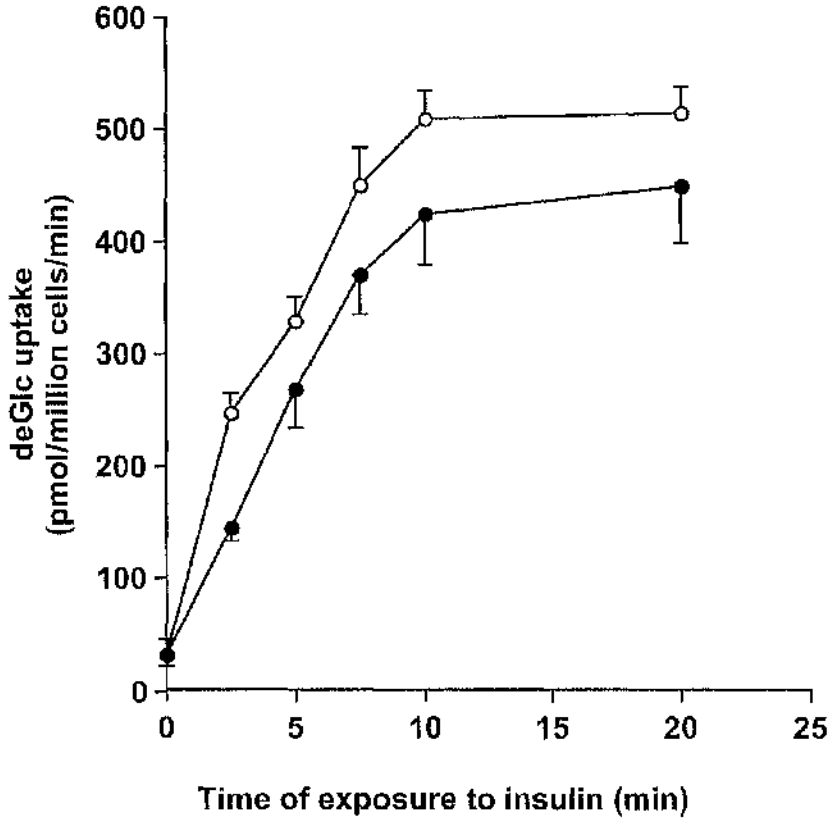
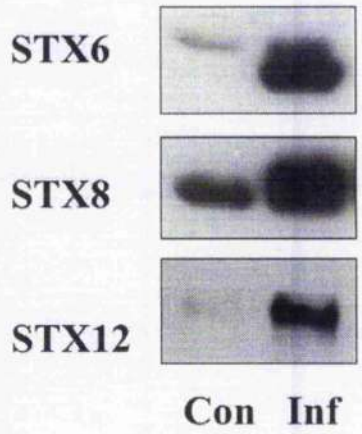

600
500
400
300
200
100
0
deGlc uptake
(pmol/million cells/min)
0
5
10
15
20
25
Time of exposure to insulin (min)

Figure 3.10 Syntaxin 6 slows the reversal of insulin-stimulated deGlc transport

Day 6 3T3-L1 adipocytes were infected with either empty virus or virus expressing the cytosolic domains of Syntaxins 6, 8 and 12, and assayed 48 h later. Figure 3.10a shows overexpression of Syntaxins 6, 8 and 12 in lysates prepared from infected cells.

Before assay, cells were incubated in serum-free medium for 2 h. Cells were stimulated with 100 nM insulin for 20 min, and then either assayed immediately or washed with KRM to induce reversal of insulin-stimulated deGlc transport for the times shown. DeGlc was assayed and Figure 3.10b shows that the rate of reversal of insulin-stimulated deGlc transport was significantly slower in cells overexpressing Syntaxin 6, compared with the empty virus cells or cells expressing either Syntaxin 8 or Syntaxin 12.

a


STX6
STX8
STX12
Con
Inf

b

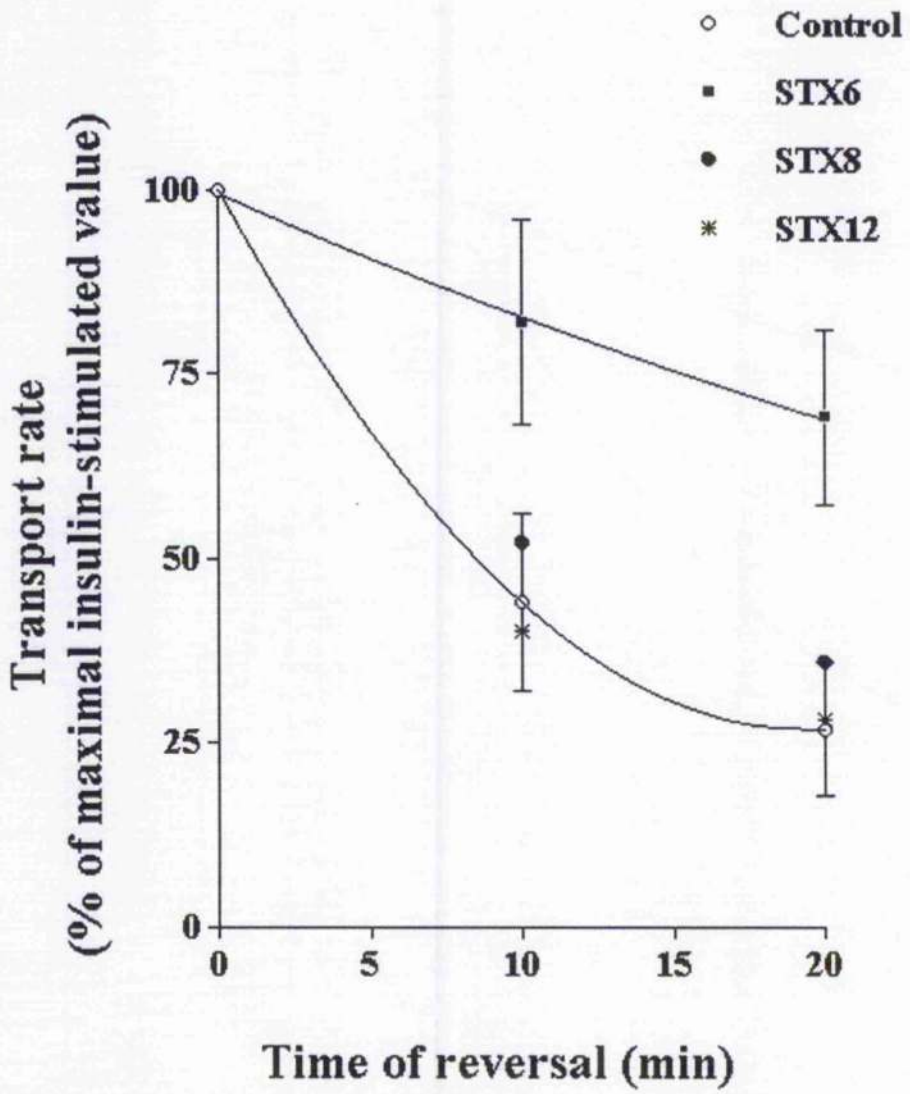

Control
STX6
STX8
STX12
Transport rate
(% of maximal insulin-stimulated value)
100
75
50
25
0
0
5
10
15
20
Time of reversal (min)

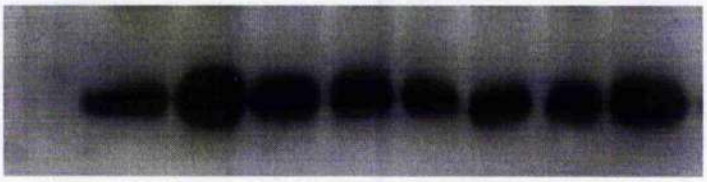
Figure 3.11 Effect of overexpression of Syntaxins 6, 8 and 12 on secretion of ACRP30

Fig 3.11a shows a typical immunoblot of secretion of ACRP30 from 3T3-L1 adipocytes overexpressing Syntaxins 6,8 and 12.

The blots from three independent experiments were scanned and the results combined

3.11b shows the results from the denstometric scans of the ACRP30 blots ± S.D.

a



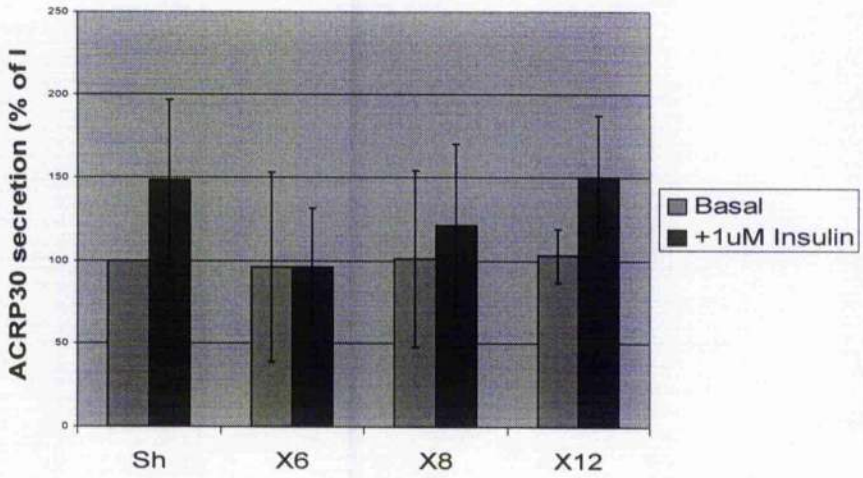
ACRP30

- + - + - + - +

Insulin

Sh X6 X8 X12

b



Chapter4 Role of GTPases in ACRP30 secretion

4.1 Introduction

The impairment of secretion of ACRP30 by ablation of transferrin receptor positive endosomes (Figure 3.6) strongly suggests that secretion of this protein involves trafficking to the cell surface via endosomes. Here I have explored the pathway of ACRP30 secretion from adipocytes.

The control of traffic in the endosomal system is regulated by a range of small molecular weight GTPases of both the Rab and ADP ribosylation factor, or Arf, classes (Fischer et al., 1994; Zerial and McBride, 2001; Donaldson and Jackson, 2000). Arf6, the sole member of the Class III Arf protein family has been localised to endosomes, endosomal tubular-vesicular structures and the PM (D'Souza-Schorey et al., 1998; Gaschet and Hsu, 1999), where there is evidence for its involvement in the regulation of such diverse membrane trafficking events as clathrin-dependent and clathrin-independent endocytosis, endosome recycling, regulated secretion and actin remodelling. Many of these functions are likely to be a result of activation of PIP(5)K and phospholipase D (PLD) and a resulting increase of phosphatidylinositol (4,5)-bisphosphate (Honda et al., 1999; Brown et al., 2001). Cell migration membrane ruffling and phagocytosis are all thought to depend on Arf6-regulated actin remodelling (D'Souza-Schorey and Chavrier, 2006). Arf6 has previously been implicated in the secretion of adipsin (Yang and Mueckler, 1999) a further reason for investigating its involvement in ACRP30 secretion.

Ullrich *et al.* have shown that Rab11 is associated with the pericentriolar recycling endosome, (Ullrich et al., 1996) and Rab11 mutants modify the morphology of this compartment, and have been shown to influence transport between the early endosome and the TGN (Wilcke et al., 2000). Rab11 has also been localised to GLUT4-containing vesicles and is thought to be involved in the endosomal recycling and sorting of GLUT4 (Kessler et al., 2000).

Because of the involvement of Rab11 and Arf6 in endosomal trafficking and the existence of adenoviruses that drive the overexpression of various mutants of these two proteins, opportunities exist to explore the pathway of secretion of ACRP30 from 3T3-L1 adipocytes. In order to define the role of Rab11 in the trafficking of ACRP30, I overexpressed a GDP-restricted mutant of Rab11 (Rab11S25N) in 3T3-L1 adipocytes and observed a significant reduction in the secretion of ACRP30. Studies of GTPase function often use dominant negative or constitutively active mutants. Dominant negative mutants

cannot be activated because they are unable to bind GTP and constitutively active mutants are lacking in the GTPase that is necessary to hydrolyse GTP, resulting in mutants that are locked in the GDP or the GTP bound conformation. Arf6 T157A can bind and release GTP more quickly than the wild type and is therefore called a fast cycling mutant. It has been shown to induce actin rearrangements in HeLa cells and increases motility of MDCK cells (Santy, 2002) Using a fast-cycling mutant of Arf6 , Arf6-T157A (Santy, 2002), I provide further evidence for a role for Arf6 in constitutive secretion from cultured adipocytes. The data presented here are consistent with a role for Rab11 in this secretion and suggests that ACRP30 secretion arises mainly through recycling TfR-positive endosomes and involves Arf6 function.

4.2 Experimental Procedures

4.2.2 Assay for secretion of ACRP30

Mature 3T3-L1 adipocytes were grown in 12-well plates. At days 8-11 post-differentiation, cells were serum-starved for 2h in serum-free DMEM at 37°C. Where the procedure was carried out in 10cm plates, cells were washed three times with 10mL Krebs Ringer Phosphate buffer (KRP) and at time t=0 this was replaced with 5mL fresh KRP± 1μM Insulin at 37°C. At each time point, 0.5mL KRP was removed to an eppendorf. In the case of 12 well plates KRP was replaced with 0.5mL fresh KRP± 1μM Insulin at 37°C, and at various time points, indicated in each experiment, this was removed to an eppendorf. In the 12 well plates, each condition was performed in three neighbouring wells, and each experiment was performed three times.

In both cases the 0.5mL sample was TCA precipitated (section 2.7), the pellet was resuspended in 50μL SDS-PAGE sample buffer containing 20mM DTT (section 2.2.2) and 20μL was loaded onto a 10% SDS-PAGE gel (section 2.13), and electroblotted onto nitrocellulose. Western blots were blocked with 5% Marvel in PBS for 1h at room temperature, followed by incubation with rabbit anti-ACRP30 anti-serum diluted 1:2000 in PBS containing 1% Marvel for 1h at room temperature. After thorough washing with several changes of PBS, blots were incubated with Horseradish Peroxidase (HRP)-conjugated donkey anti-rabbit IgG (whole molecule), diluted 1:1000 in PBS containing 1% Marvel for 1h at room temperature. Following thorough washing with several changes of PBS, the antigen-antibody complex was detected with enhanced chemiluminescence (section 2.15.1) and visualised by autoradiography. The autoradiographs were scanned and

quantified denstitometrically, using the image-processing programme Image J. The value obtained for each was expressed as a percentage of the value for the control band in each experiment, and the results shown are the combination of three experiments (unless stated otherwise).

4.2.2 Infection of 3T3-L1 cells with adenoviruses designed to drive the overexpression of Rab11S25N, Arf6T157A, wild type Arf5 and Arf5T31N

Day 8 mature 3T3-L1 adipocytes, grown in 12-well culture plates were infected with adenovirus at a moi of 50 pfu/cell. The virus was added to the cells in 0.5mL serum-free DMEM for 4h, after which time 0.5mL DMEM containing 10% (v/v) FCS was added to each well. Cells were incubated with virus for 48h, before being assayed for secretion of ACRP30.

Cells infected with the Arf6T157A adenovirus and cells infected with the adenovirus containing the empty pShuttle vector were lysed 48 h after infection, in 500 μL per well of HES buffer. Lysates were homogenised 10X with a needle and centrifuged at 4°C for 30 min at 10,000 x g. Proteins were precipitated as described in Section 2.7 and re-suspended in 100 μ l of 1X SDS-PAGE sample buffer. 10 μL was loaded on a 12% gel and immunoblotted with rat anti-HA antibody diluted 1:1000 in PBS containing 1% Marvel for 1h at room temperature. This revealed the presence of a band of just over 25 kDa in the Arf6T157A virus infected cells, which was absent from the pShuttle virus infected cells.

Arf5wt and Arf5T31N adenoviruses both express GFP and confirmation of expression of these two proteins was carried out microscopically. When > 60% of cells infected expressed GFP, the experiments proceeded.

4.2.3 Ablation of the Transferrin receptor-positive endosomal compartment in 3T3-L1 adipocytes overexpressing Rab1125N

Day 8 3T3-L1, adipocytes grown in 12 well culture plates, were infected with an adenovirus overexpressing the GDP-locked Rab11 mutant Rab11-S25N, or containing the empty pShuttle vector at an moi of 50 pfu/cell. 48h after infection, half the plates were treated to ablation of the recycling endosome compartment see Section 2.8, after which

cells were assayed for secretion of ACRP30. 1μM Insulin was added at t = 0 of the secretion assay to experimental plates only.

Cells infected with the Rab11-S25N adenovirus and cells infected with the adenovirus containing the empty pShuttle vector were lysed 48 h after infection, in 500 μL per well of HES buffer. Lysates were homogenised 10X with a needle and centrifuged at 4°C for 30 min at 10,000 x *g*. Proteins were precipitated as described in Section 2.7 and re-suspended in 100 μl of 1X SDS-PAGE sample buffer. 10 μL was loaded on a 12% gel and immunoblotted with rabbit anti-myc antiserum diluted 1:1000 for 1h at room temperature. This revealed the presence of a band of just over 20 kDa in the Rab11-S25N virus infected cells, which was absent from the pShuttle virus infected cells.

4.3 Results

4.3.1 Rab 11S25N overexpression inhibits secretion of ACRP30

3T3-L1 adipocytes were infected with empty adenovirus or adenovirus containing Rab11S25N. In Rab11S25N virus-infected cells, both basal and insulin-stimulated secretion of ACRP30 were significantly inhibited by over-expression of Rab11S25N as shown in Figure 4.1. A small fraction of ACRP30 secretion was still evident after expression of Rab11S25N and this was sensitive to ablation of the TfR positive endosomes, suggesting that Rab11S25N is unable to completely inhibit trafficking through the recycling endosomes.

4.3.2 Effects of overexpression of a Fast Cycling Arf6 Mutant on ACRP30 secretion

3T3-L1 adipocytes were infected with empty virus or virus containing the fast-cycling Arf6-T157A mutant. Cells were then assayed for secretion of ACRP30 Over-expression of Arf6-T157A stimulated basal secretion of ACRP30. Insulin treatment of virus infected cells resulted in reduction of secretion (Figure 4.2).

4.3.3 Effects of overexpression of wild type Arf5 and a GDP-locked mutant of Arf5 on ACRP30 secretion

Arf5 is a member of a group of soluble Arfs that are thought to be involved in the recruitment of coat proteins to membranes during membrane trafficking (Kahn et al., 2005), and, unlike Arf6, is thought to function in the Golgi and ER. Arf5 is a class II Arf and it is suggested that these Arf may have multiple and overlapping functions. In order to investigate any possible role for Arf5 in adipocyte secretion, I infected 3T3-L1 adipocytes with adenovirus vectors designed to overexpress Arf5 and the GDP-locked Arf5 T31N. 48 h post-infection cells were assayed for secretion of ACRP30.

Figure 4.3 is a representative blot which shows that overexpression of the wild type Arf5 and the GTP-hydrolysis-deficient Arf5 T31N has no effect on secretion of ACRP30.

4.4 Discussion

Rab11 is thought to control recycling between endosomes and the PM. To find out whether this GTPase was involved in ACRP30 secretion, I over-expressed a GDP-restricted mutant, Rab11-S25N in 3T3-L1 adipocytes and examined the effect on secretion. Figure 4.1 shows that expression of the mutant significantly reduced basal and insulin stimulated ACRP30 secretion. This result suggests that, in common with GLUT4, insulin-stimulated secretion of ACRP30 is Rab11 controlled. I have previously suggested that ACRP30 and GLUT4 do not traffic to the cell surface using the same regulated route (see Chapter 3). The involvement of Rab11 may however be a common feature of the insulin-stimulated secretion of these two proteins. If secretory traffic from recycling endosomes is under the control of insulin, this could be the point at which Rab11 controls the trafficking of ACRP30 and GLUT4. This is further evidence to support the involvement of recycling endosomes as an intermediate in trafficking of proteins to the cell surface in 3T3-L1 adipocytes.

Arf6 has also been implicated in the regulation of trafficking in endosomes, and its involvement in the secretion of adipsin from 3T3-L1 adipocytes has been demonstrated. Yang and Mueckler showed that overexpressing a dominant negative XTPase mutant of Arf6 in 3T3-L1 adipocytes inhibited basal and insulin stimulated secretion of adipsin from these cells (Yang and Mueckler, 1999). I have shown further evidence of a role for Arf6 in secretion from adipocytes by overexpressing the fast cycling Arf6 and demonstrating a stimulation of secretion of ACRP30 in the absence of insulin (Figure 4.2). Treatment with

insulin however, strongly inhibits secretion. A previous study of the role of Arf6 in membrane traffic, looked at the effects of overexpressing the mutant Arf6 Q67L in HeLa cells (Brown et al., 2001). This GTP-restricted mutant induced the formation of PIP_2-enriched, actin coated vacuolar structures that were unable to cycle back to the PM, implicating PIP_2 in trafficking through the Arf6 endosomal recycling pathway, and consistent with the role of PIP 5-kinase as a downstream effector of Arf6. This result was mimicked by over-expression of PIP 5-kinase, consistent with earlier evidence pointing to PIP 5-kinase as a downstream effector of Arf6 and demonstrating that alterations of membrane lipid composition, induced by over-expression of Arf6 can shut down traffic through the Arf6 dependent endosomal pathway. Here I have found that over-expressing the fast cycling mutant of Arf6 increased basal secretion of ACRP30 but this secretion was strongly inhibited by the addition of insulin. This suggests that under basal conditions Arf6 T157A induces PIP_2 production through the activation of PIP 5-kinase and insulin stimulation causes the further activation of Arf6 T157A, raising PIP_2 levels sufficiently to cause formation of these large vacuoles identified by Brown and co-workers (Brown et al., 2001). Therefore overexpressing T157A under basal conditions, may induce PIP_2 production enough to see the increase that I see in Figure 4.2. Stimulation with insulin, however could raise PIP_2 levels to a point where they become inhibitory, and traffic through the Arf6 regulated pathway shuts down, thus explaining the inhibition of secretion seen in the insulin-stimulated samples in Figure 4.2.

Arf6 is a regulatory molecule in the secretory pathway. It may be involved in the control of an intracellular pool of PIP_2 (Aikawa and Martin, 2003), through the activation of PIP5-kinase. Arf6 also has a role in recycling between the endosomal compartments and the PM (Brown et al., 2001) and is thought to control actin dynamics at the periphery (D'Souza-Schorey and Chavrier, 2006). I have presented further evidence that Arf6 is an important regulator of secretion in adipocytes.

Arf5 is a member of the Class II Arfs that are mainly involved in trafficking in the Golgi and ER. Overexpression of wild type Arf5 and the guanine nucleotide exchange factor deficient Arf5T31N had no effect on ACRP30 secretion in 3T3-L1 adipocytes. A previous study has shown, using siRNA, that knocking down Arf5 alone had no effect on morphology of the Golgi or endosomes or localisation of coat-proteins. Double knock-downs however, involving Arf4 and Arf5, showed enhanced Golgi localisation of the KDEL receptor and a 45% impairment of Tfn recycling (Volpicelli-Daley et al., 2005). This apparent need for both Arf4 and Arf5 to be knocked down in order to see functional

effects could explain why I saw no effect on trafficking of ACRP30 in cells overexpressing Arf5T31N.

Figure 4.1 Effect of overexpression of Rab11S25N on ACRP30 Secretion

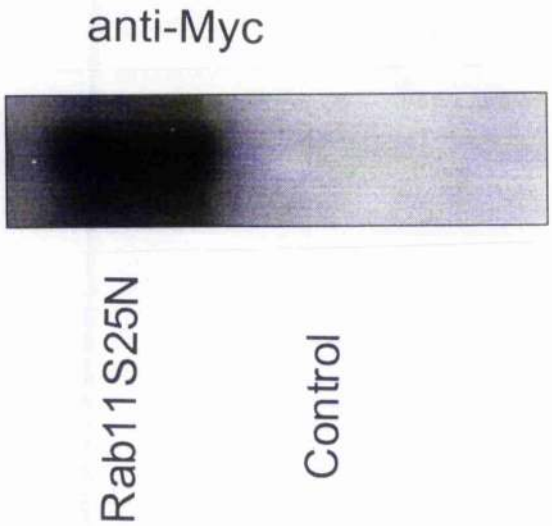
4.1a Revcals the presence of a band of just over 20kDa in the Rab11-S25N virus –infected cells, which is absent from the pShuttle virus infected cells.

4.1b Shows that in Rab11S25N virus-infected cells, both basal and insulin-stimulated secretion of ACRP30 are significantly inhibited.

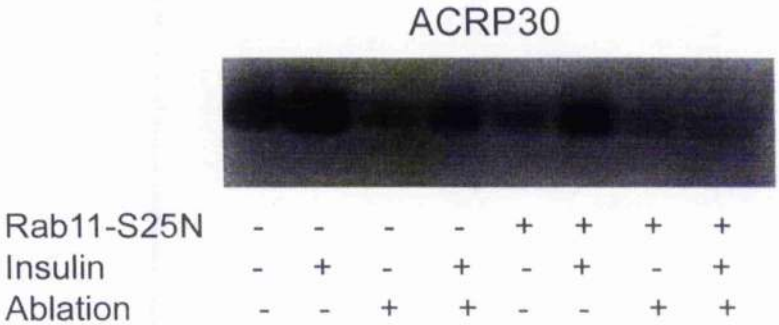
The blots from three independent experiments were scanned and the results combined

4.1c shows the results from the denstometric scans of the ACRP30 blots ± S.D.

a


anti-Myc
Rab11S25N
Control

b


ACRP30
Rab11-S25N - - - - + + + +
Insulin - + - + - + - +
Ablation - - + + - - + +

c

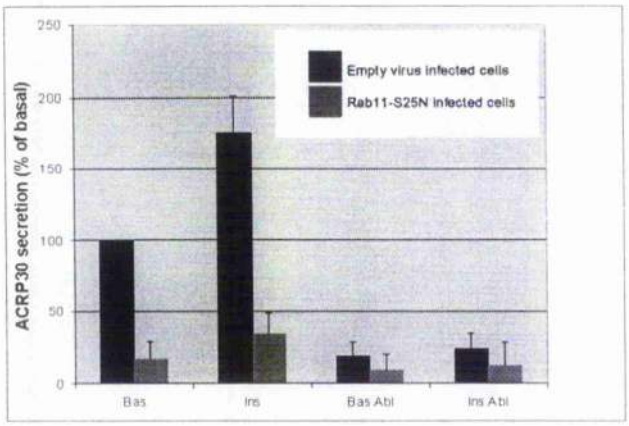

ACRP30 secretion (% of basal)
250
200
150
100
50
0
Empty virus infected cells
Rab11-S25N infected cells
Bas
Ins
Bas Abl
Ins Abl

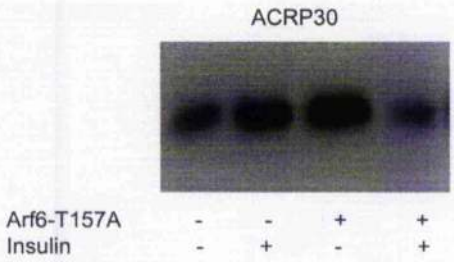
Figure 4.2 Effect of overexpression of a fast cycling Arf6 mutant on secretion on ACRP30 secretion in 3T3-L1 adipocytes

Figure 4.2a shows that overexpression of the fast-cycling Arf6 mutant stimulates basal secretion of ACRP30, and that insulin-stimulation of these cells results in inhibition of secretion.

The blots from three independent experiments were scanned and the results combined

4.2b shows the results from the densitometric scans of the ACRP30 blots ± S.D.

a


ACRP30
Arf6-T157A - - + +
Insulin - + - +

b

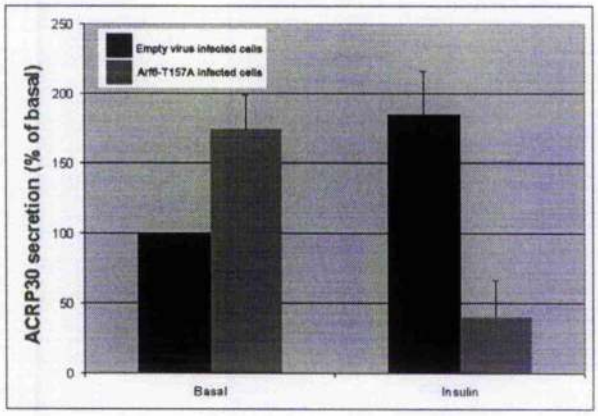
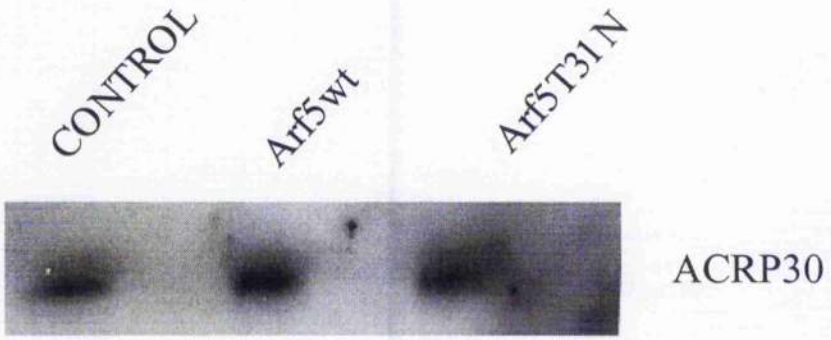

ACRP30 secretion (% of basal)
250
200
150
100
50
0
Empty virus infected cells
Arf6-T157A infected cells
Basal
Insulin

Figure 4.3 Effect of overexpression of Arf5 and Arf5 T31N on ACRP30 secretion in 3T3-L1 adipocytes

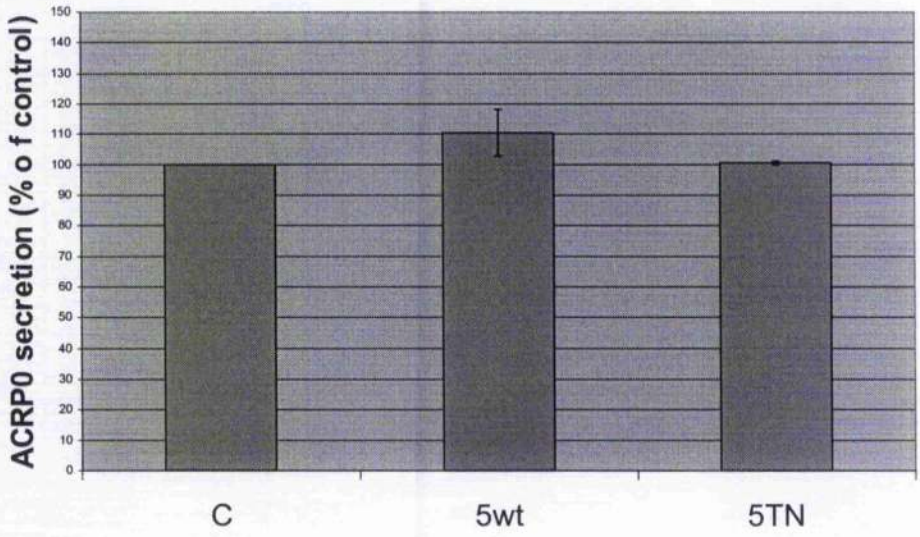
Figure 4.3a shows a typical blot of ACRP30 secretion in cells infected with either pshuttle, Arf5wt or Arf5T31N. Overexpression of the virus has no effect on secretion of ACRP30

Fig4.3b Two independent experiments were scanned and densitometrically measured. n=2 ± S.D

a


CONTROL
Arf5wt
Arf5T31N
ACRP30

b


ACRP0 secretion (% o f control)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
C
5wt
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Chapter 5 Proteins secreted by 3T3-L1 adipocytes – A Proteomic Screen

5.1 Introduction - Secreted proteins from 3T3-L1 adipocytes

Obesity is one of the major health problems facing the world in the 21st century, and is defined by excess body fat (> 30% of body weight made up of adipose tissue). This adipose tissue is an endocrine organ that secretes a variety of metabolic or differentiation-controlling hormones called adipokines. Many novel secreted proteins with involvement in glucose and lipid metabolism have been identified recently, but the complete picture of proteins secreted from fat cells has not been described. In the search for new adipocyte-secreted proteins many different approaches have been adopted; adipocyte-specific genes have been screened, resulting in the discovery of resistin (Steppan et al., 2001a); 1D-electrophoresis and 2-D-electrophoresis combined with liquid chromatography-based separation and tandem mass spectrometry (MS/MS). Using a combination of 1D gel-electrophoresis and nanospray tandem mass spectrometry to analyse the differential expression of secreted proteins between 3T3-L1 pre-adipocytes and day 9 mature adipocytes Kratchmarova and co-workers identified four secreted molecules that had not been previously shown to be expressed during adipocyte differentiation (Kratchmarova et al., 2002). In another study (Chen et al., 2005) adopted a quantitative approach, using 2D LC-MS/MS to examine the secreted proteome from rat primary adipocytes. They compared the difference in secreted proteins between basal and insulin-stimulated cells and identified 84 proteins that they believed to be adipokines, 53 of which had not previously been identified as such.

The key aims of this study are to identify new adipokines and to characterise them for their biological role and with this aim, I have chosen 3T3-L1 adipocytes as a source of secreted proteins. I have carried out a screen of the secreted proteins of mature day 10 3T3-L1 adipocytes with a view to identifying novel secreted proteins from these cells. This clonal cell line is a well-accepted model for primary adipocytes and the cells are known to secrete resistin, ACRP30, and adipsin (Millar et al., 2000).They are an excellent model system for studying secretion in adipocytes. Since the adipocytes are differentiated from pre-adipocytes, they provide a valuable source of reproducible material from which conditioned medium can be collected.

This study analyses the total secreted proteome from 3T3-L1 adipocytes by using 2D Liquid Chromatography, coupled to tandem mass spectroscopy. This method separates intact soluble proteins by liquid chromatography, before subjecting them to the proteolytic

digestion that precedes mass spectroscopy, and was chosen as an approach for this study because it allows a greater scope for inclusion of all proteins than some of the earlier approaches. 2D-PAGE (polyacrylamide gel electrophoresis), coupled to mass spectroscopy, a common approach for proteomic analysis, was tried and rejected for this study because of the problems encountered with solubilising the whole sample in the first dimension sample buffer. Many of the proteins secreted by 3T3-L1 adipocytes are heavily glycosylated and the detergent–free buffer first dimension sample buffer proved ineffective at completely solubilising the sample. Since one aim of the study was to identify as many proteins as possible form the whole secreted proteome, it was necessary to find a way of including the whole sample in the experiment. This was achieved by modifying the sample preparation method used by Kratchmarova and co-workers (see section 5.2.1 for details). Using this method, I have identified 67 secreted proteins from the Mascot Search results, 25 of which have not previously been identified as adipocyte-secreted proteins.

Six of these potential new adipocyte-secreted proteins have been screened for their effects on insulin sensitivity in 3T3-L1 adipocytes, on pre-adipocyte conversion to adipocytes and on adhesion of U937 cells to Human Umbilical Vein Endothelial cells (HUVECs).

5.2 Experimental Procedures

5.2.1 Preparation of secreted protein sample for Proteomic analysis

The sample was prepared from the conditioned medium of 3T3-L1cells using a modified version of the method used by Kratchmarova *et al* (Kratchmarova et al., 2002). Day 10 3T3-L1 adipocytes were washed six times using 10ml serum free DMEM per 10cm culture dish. The final wash was with serum-free DMEM without phenol red, and the cells were left in 8ml serum free DMEM without phenol red for 10 hours. Conditioned medium was removed and passed through a 0.2μm filter, after which it was dialysed in a dialysis cassette with a molecular weight cut off 3500 kDa against distilled H_2O. The dialysed sample was concentrated in a vacuum centrifuge but not to dryness. When the sample was concentrated to approximately 100μg in less than 100μl, it was used for 2D LC-MS/MS.

1X 10cm plate of day 10 3T3-L1 adipocytes was washed 6X with 5ml of serum free DMEM, before being incubated in 2.5ml serum free DMEM at 37°C for 2h. The medium was collected and the proteins were precipitated with TCA see Section 2.5. The protein

pellet was re-suspended in 20µl High Urea sample buffer and 10µl sample was loaded on each of two lanes of a 12% SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue stain (see Section 2.16).

5.2.2 Proteomic analysis

Trypsin digest was performed at 37°C overnight in 25 mM ammonium bicarbonate, 0.1% n-octyl-glycoside. Tryptic peptides were dried, resolubilized in 0.5% formic acid and fractionated by nanoflow HPLC on a C18 reverse phase column, eluting with a continuous linear gradient to 40% acetonitrile over 20 minutes. Eluate was analysed by online electrospray tandem mass spectrometry using a Qstar Pulsar XL (Applied Biosystems).

Mass spectrometric analysis was performed in IDA mode (AnalystQS software, Applied Biosystems), selecting the four most intense ions for MSMS analysis. A survey scan of 400–1500 Da was collected for 3 sec followed by 5 sec MSMS scans of 50–2000 Da using the standard rolling collision energy settings. Masses were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the Mascot (Matrix Science) search engine for protein identification.

5.2.2 Interpretation of Mascot Search Results

Mascot search results were initially divided into proteins that were known to be secreted and proteins not known to be secreted. From the list of proteins not known to be secreted, I removed those that were known not to be secreted i.e. known intracellular proteins. The proteins remaining (possible secreted proteins) were analysed using various sequence prediction programmes, and secreted protein databases, until a final list of likely secreted proteins was developed.

Only those proteins with two peptide hits, each with a probability based MASCOT score greater than the cut-off score that indicates homology or identity, were included in the list of secreted proteins. Proteins with only one bold red *or faint red **peptide hits above the cut-off score of 40 were manually validated by checking several parameters in the results.

*Indicating the highest score and first appearance in the list for this particular peptide.

** Indicating the highest score but not the first appearance for this peptide.

The mass spectrum, included for each protein in the MASCOT search results, displays all the labelled, matched fragment ions. Also displayed in the Mascot results are the matched fragment ions in tabular form. With the "one peptide" protein hits, I checked that they displayed a reasonably full set of matched y or b ions across the length of the peptide. Where there was not a good set of y or b ions I further checked the sequence by blast searching the section of the sequence that was well matched for ions. When the blast search agreed with the original protein identification, I took this as further validation of the protein. Where appropriate i.e. when there were amino acids present in the sequence whose cleavage would result in strong immonium ions I further validated the sequence by checking for the presence of immonium ions on the spectrum. The 16 proteins that were further validated in this way have been included in the list of secreted proteins but are listed as manually validated (Table 5.1).

5.2.2 Immunoblotting of serum proteins

Plasma taken from lean and fatty Zucker rats was re-suspended in 3X SDS-PAGE sample buffer containing 50 mM DTT and 5 µL was loaded on a 10% SDS-PAGE gel. Gels were immunoblotted for HSP90Cα, HSP70 and orosomucoid. Using rat anti HSP90α diluted 1:150,000, rabbit anti-HSP70 diluted 1:100,000 and Chicken anti-orosomucoid diluted 1:500 .HSP90α blots were subsequently incubated horseradish peroxidase conjugated anti-rat IgG 1:1000; HSP70 blots were incubated with horseradish peroxidase conjugated anti-rabbit IgG 1:1000 and orosomucoid blots were incubated with horseradish peroxidase conjugated anti-chicken IgY 1:1000. All incuabtions were carried out in PBS containing 1% Marvel, and all for 1h at room temperature.

In the case of blot a., showing HSP90α, the sample was diluted 1:10 in PBS before re-suspension in sample buffer, and the samples were separated by SDS-PAGE and Western blotted, according to sections 2.14 and 2.15.

As a loading control, equal amounts of serum from lean and fatty rats were separated by SDS-PAGE and blotted with horseradish peroxidase linked anti-rat IgG, diluted 1:1000 in PBS, containing 1% Marvel for 1h at room temperature.

5.2.3 Detection of secreted proteins in the 3T3-L1 conditioned medium by immunoblotting

3T3-L1 adipocytes were grown on 12 well culture plates and secretion assay was carried out at day 8 post-differentiation as described in section 2.5. Cells were incubated in KRP ± 1μM Insulin for 10min, and secreted proteins were TCA precipitated as described in section 2.7. Antibody incubations were carried out as in section 5.2.2.

5.2.4 2-deoxyglucose uptake by 3T3-L1 cells

Triplicate wells of 12 well plates of day 8 3T3-L1 adipocytes were incubated overnight in serum-free DMEM containing defined concentrations of recombinant proteins. Cells were washed 3X in KRP and incubated with or without insulin (1nM or 100nM) for 15min at 37°C. Glucose transport was measured by the assay of 2-deoxy-D-glucose as described in section 2.7, adding an equivalent of 25uCi [^{3}H] per well. Data was combined from three experiments for each protein except autotaxin and LRG1 where two experiments were combined.

5.2.3 Oil Red O Assay

3T3-L1 adipocytes were grown on 12 well culture plates and treated with defined concentrations of recombinant proteins, on days 0, 2, 4 and 6 of differentiation, where day 0 is the first day of the addition of IBMX, dexamethasone and insulin. On day 8 of differentiation cells from duplicate wells were fixed in 10% formaldehyde and stained with Oil red O as described in section 2.8. Oil Red O stain was extracted from the cells and the OD_{520} was measured.

5.2.4 3T3-L1 Differentiation Assay

3T3-L1 adipocytes were grown on 12 well culture plates and treated with PF4 (2μg/mL), Nm23 (10ng/mL), LRG1 (5μg/mL), orosomucoid (5μg/mL), HSP90α (5μg/mL), and HSP70 (5μg/mL) on days 0, 2, 4 and 6 of differentiation, where day 0 is the first day of the addition of IBMX, dexamethasone and insulin. On day 8 of differentiation cells from duplicate wells were lysed in 125 μL per well of SDS Lysis Buffer. Cell lysates was mixed with 3X SDS-PAGE sample buffer containing 50 mM DTT and sample were loaded on a 10% SDS-PAGE gel and immunoblotted for PPARγ and CEBPα.

Blots were incubated with either mouse anti-PPARγ diluted 1:200, rabbit anti-C/EBPα 1:200, or mouse anti-GAPDH 1:5000 in PBS containing 1% Marvel for 1h at room temperature.

This blot shows results from a single experiment. For each condition, at least two wells were combined to make the lysates.

Equal loading was determined by immunoblotting for GAPDH.

5.2.5 Monocyte adhesion to HUVECs

10^4 HUVECs were seeded into each well of a 24-well plate and grown at 37°C for 48h. In order to remove any potential inflammatory effect caused by endodtoxin contamination of the recombinant proteins, they were pre-incubated with 2μg/mL Polymyxin B sulphate for 30m The cells were then incubated for 4h with the recombinant proteins indicated in the Figure legend, and the cells overlaid with U937 promonocytic cells. U937 cells were allowed to adhere for 1h, after which time the monolayers were washed to remove non-adhering monocytes and adherent cells were counted. At least 300 HUVECs were counted in 9 separate fields (3 each from 3 different experiments) and the number of adherent U937 cells was calculated per 100 HUVECs for any given treatment.

5.3 Results of proteomic analysis

5.3.1 Proteomic analysis of secreted proteins

We performed 2D LC-MS/MS analysis to identify the secreted proteome of mature 3T3-L1 adipocytes. The sample was prepared from the conditioned medium of 3T3-L1cells using a modified version of the method used by Kratchmarova *et al* (Kratchmarova et al., 2002). Kratchmarova *et al.,* suggest vacuum centrifuging the protein sample to dryness before re-suspending it in buffer for 2D LC-MS/MS. When following this protocol I experienced problems with re-suspending the sample, presumable because of the presence of heavily glycosylated proteins like collagen. By stopping the drying process at an earlier stage, the

resulting sample was more representative of the entire secreted proteome as there was no insoluble material left behind.

Figure 5.1 shows a 10% SDS-PAGE gel, stained with Coomassie brilliant blue of a typical set of secreted proteins from mature 3T3-L1 adipocytes.

401 candidate proteins were returned from the Mascot search results. A number of which were non-secreted proteins that had leaked into the conditioned medium from dead cells. To ensure that only secreted proteins were identified, the candidate proteins were validated by one or more sequence prediction servers. Signal P 3.0 predicts the presence and location of signal peptide cleavage sites in amino acid sequences, PSORT is a programme for the prediction of cellular localisation of a protein and Secretome P 2.0 produces predictions of non-classical i.e. not signal peptide triggered protein secretion. Using a combination of these prediction programmes and previously published data on each of the proteins, a set of 67 secreted proteins was compiled, and these are listed in Table 5.1.

An example of a protein hit that was manually validated is TSC-36/FRP (gi 2498391), which has an ions score of 52. This protein scored one bold red * peptide hit with an ions score of 43. Figures 5.2a and 5.2b show the mass spectrum and the ions table respectively for this peptide. The peptide sequence is LIQWLEAEIIPDGWFSK. The table of ions for this peptide shows a broken run of y ions in the section IPDGWFSK, and a strong run of b ions in the section IQWLEAE. The shorter section of the peptide sequence with a strong sequence of b ions was compared against the *mus musculus* database in BLAST (Basic Local Alignment Search Tool) and the results indicated that follistatin-related protein was the most statistically significant match. This sequence data gave me reasonable confidence that this peptide hit was follistatin-related protein and should be included in the list of novel secreted proteins.

Several of the proteins in Table 5.1 were previously identified as being secreted from primary adipocytes. As one of my aims was to identify which of the proteins secreted by primary adipocytes were also secreted by cultured 3T3-L1 adipocytes, I compared the results of this screen with those of Chen and co workers. Table 5.2 shows those proteins that are common to both studies.

Of the 84 proteins identified by Chen and colleagues, I show here 14 that are also secreted by 3T3-L1 adipocytes. Although previous studies of the secreted proteome of 3T3-L1 cells have identified proteins in common with those secreted by primary adipocytes, there are

several that do not appear on the list of primary adipocyte secreted proteins, including some of the collagens (types I, II And VI). Chen and co workers suggest that the different profiles of secreted proteins are a likely reflection of the different biological characteristics of these two cell types. Our analysis shows that the difference in secreted proteome of the two cells types is not as great as suggested by the comparison of the data of Chen *et al* and Kratchmarova *et al*. I have found 14 proteins in common, suggesting that there are clear similarities between the two cell types. The relative distribution of proteins, according to type, varied between the two studies, as illustrated in Figure 5.3.

5.3.2 Extracellular Matrix

In addition to the proteins shown in Table 5.2, I have identified a further 25 novel proteins secreted from 3T3-L1 adipocytes cells (Table 5.3). A large proportion (39%) of these are associated with the ECM. This includes matrix proteins and those involved in regulation of the matrix and its attachment to cells. These ECM associated proteins found in my study and the study undertaken by Chen *et al* are listed in Table 5.4. Chen *et al* have suggested that these may represent proteins secreted by 3T3-L1 adipocytes during their adaptation to growth in culture. It is true that adipocytes *in vivo* are surrounded and supported by extracellular matrix proteins (Nakajima et al., 1998), which play a dynamic role in the differentiation of adipocytes and the development of adipose tissue. There is evidence that *in vivo* the ECM is much more than a structural support for cells but is in fact a more dynamic element in adipose tissue. It is known that during adipogenesis, the differentiation of a pre-adipocyte into an adipocyte is dependent on the cell changing shape and it is thought that this shape change is dependent on remodelling of the matrix by MMPs (Spiegelman and Ginty, 1983; Bouloumie et al., 2001). ECM and ECM-associated proteins are involved in processes such as cell movement, tissue remodelling and intracellular signalling (Damsky et al., 1992; Mott and Werb, 2004). The extracellular matrix also acts as a pool of proteins that can interact with cells and activate signalling. Below I comment on some of those proteins listed in Table 5.4.

Fibronectin Laminin and **Vitronectin** are adhesive proteins found in the basal lamina. They attach cells to the supportive matrix via specialised domains. Fibronectin has several binding domains specific for matrix molecules like collagen, and others specific for cell surface receptors (integrins) that are known to activate intracellular signalling cascades (Potts and Campbell, 1994). Attachment of these ECM molecules to cell surface integrins is known to activate NFκB, a transcription factor that plays a central role in many cellular responses e.g. inflammation, migration, differentiation and development and ECM

stimulation of NFκB via integrins is necessary for glucose stimulated insulin secretion in pancreatic B cells (Hammar et al., 2005; Mott and Werb, 2004). Proteolytic fragments of **fibronectin** can promote adipocyte differentiation and induce cell migration (Mott and Werb, 2004). **Decorin** (connective tissue) and **biglycan** (bone and cartilage) are both small leucine rich proteoglycans (SLRP) that bind collagen and C1q and act as inhibitors of activation of the complement cascade and C1q dependent cytokine production (Groeneveld et al., 2005) **Versican** is a large chondroitin sulphate proteoglycan, found in many tissues. Versican has anti-adhesive activity in the globular domain but the carboxy terminal is involved in the formation of focal adhesions via binding to integrin. Versican also binds to molecules on the surface of inflammatory leucocytes and has been shown to bind, through its chondroitin chains, chemokines involved in inflammation (Hirose et al., 2002).

Perlecan is found in all basement membranes. Its GAGs are mainly heparan sulphate and it has a domain that is homologous to the LDL receptor (Murdoch et al., 1992)

5.3.3 Extracellular matrix associated proteins

In addition to the proteins that make up the extracellular matrix, there is a range of bioactive molecules in the pericellular matrix, which along with the extracellular matrix proteins affect cell behaviour. Some examples of these proteins were identified in my screen (Table 5.4) and are discussed below.

Metalloproteinases are proteases that act on proteins of the ECM. As well as being responsible for remodelling and degradation of the matrix during the progression of certain diseases including invasion of tissue by cancer cells, they can release cryptic biologically active fragments from ECM molecules (Matrisian, 1992). **MMP2** exposure of an epitope on collagen IV is required for angiogenesis (Xu et al., 2001). MMP2 acts on **decorin** to release sequestered TGF-β (Imai et al., 1997). Anti-angiogenic fragments are also released from the NC1 domain of **collagen IV** (Ortega and Werb, 2002), and from **collagen XV** and **perelecan** (Ramchandran et al., 1999; Mongiat et al., 2003). Metalloproteases also release sequestered growth factors like VEGF and TGFβ from the ECM (Belotti et al., 2003; Krishnan et al., 2004), and can alter cell attachment to the ECM via their action on syndecan and dystroglycan (Mott and Werb, 2004). MMP2 can be inhibited by a fragment of **procollagen C enhancer protein** (Mott et al., 2000), and also by the action of serine protease inhibitors. **Thrombospondin 2** binds to MMP2, resulting in its endocytosis thus removing it from the ECM (Bornstein et al., 2000). Collagens I and IV, fibronectin,

laminin and vitronectin are all substrates for MMP2. **Secreted HSP-90α** is an activator of MMP2 (Eustace and Jay, 2004)

Thrombospondin 2 is thought to be involved with the disruption of focal adhesion contacts by binding to cell surface receptors (Sage and Bornstein, 1991). **Follistatin related protein/TSC-36** is also a member of the BM-40/SPARC/osteonectin family of extracellular glycoproteins, which contain an extracellular calcium binding domain and a follistatin-like domain (Shibanuma et al., 1993)

MMP2 is known to be responsible for fibronectin degradation during cell migration, mesenchymal cell differentiation, enhanced collagen affinity (by cleavage of osteonectin), increased availability of TGF-β and IGF-1. It is also reported to have an anti-inflammatory role through the degradation of IL-1 and monocyte chemo attractant protein 3 (Visse and Nagase, 2003). **Papilin** is an ECM associated protein with homology to ADAMTS metalloproteinases (Kramerova et al., 2000). **Peroxidasin** is an ECM stabilising protein with peroxidase activity (Nelson et al., 1994).

5.3.4 Novel secreted proteins

In addition to the ECM associated proteins and the proteins in common with primary adipocytes I have identified another group of proteins that have not previously been found in the secreted proteome of adipocytes. Table 5.4 is a list of potential new adipokines listed according to their biological function. This list includes protease inhibitors; proteins involved in inflammation, lipid binding and cell growth and metabolism and includes the novel ECM associated proteins.

5.3.5 Inflammation associated proteins

27% of the proteins identified in my screen are inflammation associated, including acute-phase proteins, consistent with the strong association between obesity and inflammation. Obesity has been described as a chronic low-grade inflammatory condition (Das, 2001) and the condition is accompanied by elevated levels of inflammatory cytokines such as TNF-α and IL-6, contributed, in part, by infiltrating macrophages (Weisberg et al., 2003). Adipocytes and macrophages share a marked number of biological and functional characteristics. They both express the adipocyte fatty acid binding protein aP2, PPARγ and the "macrophage" proteins TNF-α, IL-6 and MMPs and under certain stimuli pre-adipocytes can be made to differentiate into macrophage-like cells (Charriere et al., 2003).

During the progression of insulin resistance that often accompanies obesity adipocytes become enlarged, secrete more pro-inflammatory cytokines like Il-6 and TNF-α and less of the anti-inflammatory adipokine, adiponectin (Xu et al., 2003). In atherosclerotic blood vessels macrophages can also become more like adipocytes by accumulating lipids (Gargalovic and Dory, 2003).

I have identified six novel secreted proteins that have a potential role in the link between obesity and inflammation.

Cyclophilin B has an extracellular role as a strong chemo-attractant for peripheral blood lymphocytes and also supports T-cell adhesion to the ECM (Allain et al., 2002). Cyclosporin binds to Cyclophilin A, blocking its chemotactic activity and **Cyclophilin C-associated protein** is a secreted glycoprotein and a homologue of human Mac-2 binding protein. It has a role in immune regulation and inflammation and its expression in mouse macrophages is up regulated by the pro-inflammatory cytokine TNF-α (Trahey and Weissman, 1999). This is a strong indication that the cyclophilins and cyclophilin associated proteins could be involved in the inflammatory state in obesity, with secreted cyclophilins acting as chemotactic factors and cyclosporin acting as a regulator of this activity. **Platelet factor 4/CXCL4** is a pro-inflammatory chemokine secreted by platelets. It is a strong chemoattractant for neutrophils and monocytes (Deuel et al., 1981) and is also a potent inhibitor of endothelial cell proliferation (Sharpe et al., 1991). Evidence points to a possible role for **orosomucoid** as an anti-inflammatory mediator also secreted by liver, endothelial cells and leukocytes. Orosomucoid is a 41kD glycoprotein found in serum. It is an acute phase protein, also secreted by macrophages whose serum concentration increases with the obese state (Fournier et al., 2000). It binds to sites on the surface of endothelial cells. Endothelial cells treated with orosomucoid show increased metabolic activity (Sorensson et al., 2000). Plasma levels of orosomucoid are raised in the obese state (Akbay et al., 2004). **Unnamed protein produce that is homologous to ITIH4** Inter-α-trypsin inhibitor is unregulated in inflammation and may be an acute phase protein (Duan et al., 2005). **Sex limited protein** shows homology to complement C4 but has no complement activity (Nonaka et al., 1985).

5.3.6 Growth and differentiation

It is known that adipocytes are a source of factors that can affect cell growth and differentiation, including TGF-β, VEGF and IGF. I have identified four possible new adipokines that have a role in growth and differentiation in other cell types.

I Factor is a secreted nucleoside diphosphate kinase (**NDPK-A**) that is encoded by the NM23 gene and is sometimes called **Nm23 protein.** It is a differentiation inhibitory factor in the mouse myeloid leukaemia M1 cell line (Okabe-Kado et al., 1995). **Follistatin related protein.** In human lung cancer cells, FRP had negative regulatory effects on growth. FRP is the product of expression of the TGF-β induced gene TSC36. (Sumitomo et al., 2000). It inhibits proliferation and migration in vascular smooth muscle cells and inhibits invasion in human lung cancer derived cells (Johnston et al., 2000). **Leucine rich α2-glycoprotein** is a marker of granulocytic differentiation (O'donnell et al., 2002)

5.3.7 Other proteins

Selenium binding protein is similar to liver fatty acid binding protein and is known to be involved in Golgi protein transport (Porat et al., 2000). I have included it here as a putative secreted protein because it is predicted to be a non-classical secreted protein by the Secretome P server. It has previously been identified in plasma (Morrison et al., 1989). **HSP 90α** is secreted by vascular smooth muscle cells and behaves like an oxidative stress factor and activates ERK1/2 (Liao et al., 2000). It is also secreted by fibrosarcoma cells where it binds to, and inactivates MMP2, suggesting that this heat shock protein has a regulatory role in the process of cell invasion (Eustace and Jay, 2004). **HSP70** is now recognised as a secreted protein that is involved in immune regulation. Exogenously added HSP70 stimulates the production of pro-inflammatory cytokines in monocytes, through activation of NF-κB (Eustace et al., 2004a).

5.4 Functional studies on six proteins

I selected six proteins from the group of proteins that had not previously been identified as being secreted by adipocytes on which to carry out functional studies. These proteins were selected because of their potentially interesting role as adipokines, based on what was published about their effects in other experimental systems. Another criterion for selecting these particular proteins was the commercial availability of recombinant or purified proteins and suitable antibodies for the following studies.

5.4.1 Introduction

Considering the possibility that some of these novel secreted proteins might have a physiological effect on either adipocytes or other cells, I examined the effects of several of these proteins on three aspects of growth and metabolism in 3T3-L1 adipocytes. Firstly, I

measured the effects of these proteins on glucose uptake in 3T3-L1 adipocytes. Several adipokines have been shown to affect insulin signalling (Uysal et al., 1997) and it has been suggested that they do this by acting through NF-κB (Yuan et al., 2001) and the JNK/AP-1 signalling pathways (Hirosumi et al., 2002) thus enhancing the inflammatory state and decreasing insulin sensitivity.

Secondly, I examined the effects of the putative adipokines on the differentiation of pre-adipocytes into adipocytes. The protein Nm23 is an inhibitor of differentiation in certain haematopoietic cell lines (Willems et al., 2002; Okabe-Kado et al., 1995). Platelet factor 4 induces the differentiation of monocytes into macrophages (Scheuerer et al., 2000) and influences the differentiation of monocyte derived dendritic cells (Xia and Kao, 2003). The leucine-rich α-2-glycoprotein (LRG1) is an early marker of granulocyte differentiation (O'donnell et al., 2002).

I also investigated the effects of these proteins on adhesion of the human promonocytic cell line U937 to human vascular endothelial cells. The vascular endothelium forms a layer on the inside of blood vessel walls, acting as a barrier between blood components and the underlying interstitium (Risau and Flamme, 1995). Mononuclear cell invasion into the vascular wall is an important initial step in the development of atherosclerosis. Under certain conditions, including the effects of some cytokines, endothelial cells are stimulated to produce an inflammatory response that includes the expression of adhesion molecules. Integrins and other ligands on the surface of monocytes bind to these adhesion molecules and migrate into the sub-endothelial space where they transform into macrophages (Lusis, 2000). TNF-α induces the expression of adhesion molecules on the surface of endothelial cells and it has been shown that ACRP30 inhibits the surface expression of several of these adhesion molecules thus reducing monocyte adhesion (Ouchi et al., 1999). Other adipokines increase monocyte adhesion, enable migration of monocytes, or enhance their conversion into macrophages. Angiotensinogen is the precursor of Angiotensin II, which stimulates the expression of the adhesion molecules ICAM-1 and VCAM-1 in endothelial cells (Tham et al., 2002) and increases macrophage accumulation in the vascular wall (Wang et al., 2001b). Another adipokine PAI-1 is involved in thrombus formation (Sobel, 1999) and circulating levels of PAI-1 are associated with atherosclerosis (Thogersen et al., 1998). Obesity is associated with increased risk of cardiovascular disease and the association has not been fully explained at the molecular level. Orosomucoid binds to sites on the surface of endothelial cells and endothelial cells treated with orosomucoid show increased metabolic activity (Sorensson et al., 2000).

PF-4 has previously been shown to induce neutrophil adhesion to endothelial cells (Kasper et al., 2004) and has not been included in this assay.

5.4.2 Confirmation of secretion of newly identified secretory proteins from 3T3-L1 cells by immunoblotting.

To confirm the secretion of these proteins identified by proteomic analysis, secretion assays were performed as described previously. Supernatant from basal and insulin stimulated 3T3-L1 adipocytes was TCA precipitated and the resulting precipitated proteins were separated on SDS-PAGE gels, electro-blotted onto nitrocellulose membrane and detected by Western blotting. Figure 5.4 shows a panel of blots. Of the proteins tested by this method only HSP-70, HSP-90α and Orosomucoid were detectable in the supernatant from 3T3-L1 adipocytes. LRG-1 was not included in this panel because of the lack of a commercially available antibody. Platelet factor 4 and Nm23 could not be detected in supernatant secreted from 3T3-L1 adipocytes by immunoblotting. It is not clear why these proteins cannot be detected immunologically. It is possible that they are secreted at levels that are too low to be detected by the available antibodies, or that the antibodies are not of high enough affinity.

5.4.3 Detection by immunoblotting of newly identified proteins in the plasma of lean and fatty Zucker rats

Zucker rats suffer from a recessive mutation in the leptin receptor, Ob-R . Animals that are homozygous for the mutation (fa/fa) eat more, build up more fat and quickly become insulin resistant. Zucker rats are used as an animal model of genetic obesity (Stern et al., 1972). The rats are genotyped at birth and divided into lean and fatty animals before the phenotype is expressed. Plasma from lean and fatty animals was analysed by Western blotting for the presence and relative levels of the newly identified secreted proteins. Equal volumes of either lean or fatty plasma were loaded on SDS-PAGE gels and these were immunoblotted with antibodies for HSP90α, HSP70, orosomucoid, PF4 and Nm23. Figure 5.5 shows that HSP90α, HSP70 and orosomucoid can be detected by immunoblotting in the plasma of lean and fatty Zucker rats and levels of these proteins are raised in the plasma of the fatty animals. It has not been possible to detect PF4 or Nm23 in either of the serum samples. The CXC family of cytokines, of which PF4 is a member, are found in pico gram to nano gram amounts per mL in serum (Rotondi et al., 2005), a concentration that is too low to be detected by Western blotting. Human Nm23 circulates in serum at less than 10 ng/mL (Okabe-Kado and Kasukabe, 2003). PF4 and Nm23 may be secreted by

3T3-L1 adipocytes in concentrations that are too low to be detected by immunoblotting, or the antibodies may not be of a high enough affinity to detect the proteins in plasma.

5.4.4 Effects of secreted proteins on uptake of 2-deoxy-D-glucose in insulin-stimulated 3T3-L1 adipocytes

Proteins secreted by adipocytes are involved in regulating many biological processes .Of particular interest in the field of diabetes and obesity is the growing evidence of a role for certain adipokines in the development of insulin resistance.

To investigate whether the proteins play a role in insulin-stimulated GLUT4 translocation day 8 3T3-L1 adipocytes were incubated overnight in serum free DMEM containing the proteins PF4 (1 μg/mL), orosomucoid (10 μg/mL) and (30 μg/mL), Nm23 (200 ng/mL), LRG1 (150 μg/mL), HSP-90α (1 μg/mL) and (0.1 μg/mL), before assaying the cells for insulin stimulated uptake of ^{3}H 2-deoxyglucose (see Section 2.11). Fig 5.6 shows the effects of treatment with various proteins on 2-deoxyglucose uptake. The concentrations for this experiment were decided by extrapolating from serum concentrations (where known) e.g. orosomucoid circulates in the serum of normal individuals at concentrations of around 1mg/mL (Easton et al., 1962). I deduced that concentrations secreted by adipocytes would likely be much lower and decided to start with 10 and 30 μg/mL. Platelet factor 4 is an example of a CXCL cytokine and they have been found circulating in serum in pg/mL-ng/mL concentrations (Easton et al., 1962), and initial experiments were carried out with these concentrations, gradually increasing to 1 μg/mL when no effect was seen at the lower concentrations. Autotaxin was included in the initial 2-deoxyglucose uptake experiments because I originally identified it as a potential new adipokine. It was however subsequently identified by another group profiling secreted proteins during adipocyte differentiation (Wang et al., 2004), and was not therefore included in subsequent experiments.

Orosomucoid decreased insulin-stimulated glucose uptake by 20-40% in maximally stimulated cells and by up to 50% in cells stimulated with 1nM insulin. It has been shown that low-grade inflammation as measured by the presence of inflammatory serum proteins like orosomucoid and haptoglobin contributes to the regulation of insulin sensitivity and lipid metabolism (Heliovaara et al., 2005), although there is no clear evidence to suggest that these inflammatory proteins directly influence molecule transporters (Flower et al., 1993). Other members of this family include the plasma retinol binding proteins, one of which, RBP4 has recently been shown to contribute to insulin resistance in obesity and type 2 diabetes (Flower et al., 1993) . Like RBP4, elevated levels of orosomucoid are

found in obesity and its presence in serum is a predictor of the development of diabetes (Duncan et al., 2003). Secreted orosomucoid has also been detected in the urine of patients with non-insulin dependent diabetes (Ito et al., 1989), where it is a strong predictor of cardiovascular disease and mortality (Christiansen et al., 2002).

Autotaxin reduced insulin stimulated glucose uptake by 20-40%. This could be an effect of Autotaxin binding to the insulin receptor as it has been found to co-precipitate with the insulin receptor α-subunit (Belfiore et al., 1996; Maddux and Goldfine, 2000), and is an *in vitro* inhibitor of insulin receptor tyrosine kinase activity (Sbraccia et al., 1991) (Maddux et al., 1993) (Maddux et al., 1995). Autotaxin is secreted from adipocytes and its expression is up regulated in the db/db mouse model of obesity, and is up regulated in insulin resistant patients (Boucher et al., 2005), suggesting a possible link with insulin resistance. It has been reported that cellular levels of Autotaxin were inversely correlated with insulin sensitivity in skeletal muscle cells (Maddux et al., 1995).

Figure 5.6 shows that Platelet factor 4, Nm23 and LRG 1 had no significant effect on insulin stimulated 2-deoxyglucose uptake at the concentrations tested. HSP70 and HSP90α had no significant effect on insulin stimulated 2-deoxyglucose uptake at the concentrations tested.(Data not shown for HSP 70 and HSP90).

5.4.5 Effects of novel secreted proteins on the differentiation of 3T3-L1 adipocytes

The proteins PF4 (2 μg/mL), Nm23 (10 ng/mL), LRG1 (5 μg/mL), orosomucoid (5μg/mL), HSP-90α (5 μg/mL) and HSP-70 (5 μg/mL) were added to differentiating cells to ascertain whether they would have any effect on the differentiation process. Proteins were added on days 0, 2, 4 and 6 of differentiation, day 0 being the day of addition of dexamethasone, IBMX and insulin (see section 2.4.7). Differentiation was assayed by both Oil Red O staining as described in section 2.12 and western blotting of cell lysates for the expression of the transcription factor C/EBP-α, which is expressed during pre-adipocyte conversion to adipocytes. If any of these recombinant proteins acts as a differentiation inhibitor, we might expect to see a reduction in the intracellular level of C/EBPα.

Oil Red O is a suspension of Sudan Red stain, which stains lipids. When 3T3-L1 adipocytes are fixed and stained with this dye, the intracellular lipid droplets stain with Sudan red. This is a measure of differentiation, since fat cells accumulate lipids as they differentiate. After staining, the Sudan Red is extracted from the cells and the amount of

dye is measured with a spectrophotometer. Figure 5.7 shows the Sudan Red extracted from the treated cells and measured as the OD_{520}. Two recombinant proteins HSP 90α and LRG1 show a statistically significant increase in uptake of Oil Red O. This increase is very slight and is unlikely to be biologically significant.

Differentiation was also assayed by examining the expression of the transcription factors C/EBPα and PPARγ. C/EBPα and PPARγ are transcriptional activators that are expressed during adipocyte differentiation and result in the expression of adipocyte-specific proteins (Ramji and Foka, 2002). 3T3-L1 cells were incubated in the presence of various secreted proteins during the differentiation process, at the end of which cells lysates were prepared. Equivalent amounts of these lysates, as determined by blotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were analysed by SDS-PAGE and western blotting for the expression of C/EBPα and PPARγ. Figure 5.8 shows a panel of Western blots from this experiment. Lysates made from cells treated with recombinant proteins orosomucoid, Nm23, LRG1, HSP90α and HSP70 did not show an obvious increase in expression of C/EBPα. Treatment with PF4 may be causing an increase in expression of C/EBPα, through an inflammatory response. It has been shown that C/EBPα is up-regulated in some cells that have been exposed to inflammatory cytokines (Wang et al., 2001a).

5.4.6 Effects of secreted proteins on monocyte adhesion to vascular endothelial cells.

Human vascular endothelial cells (HUVECS) were incubated with either TNF-α, which evokes a pro-inflammatory response or with Orosomucoid (1mg/mL), LRG1 (0.75μg/mL), HSP70 (0.75 μg/mL) and HSP90α (0.75 μg/mL). It was decided to start with concentrations that were close to those found in serum, and gradually increase these until an effect was seen. Orosomucoid is an acute phase protein that circulates in serum and has been measured in the serum of control individuals at concentrations of between 0.235 and 3 μg/mL, and with this as a guide I decided to start with 0.75 μg/mL for both of the heat shock proteins (Njemini et al., 2003). Following a four-hour incubation with these proteins, HUVECs were assayed for their ability to bind U937 promonocytic cells. After fixing the cells with formaldehyde, monocyte adhesion was assayed by counting the number of adherent cells per 100 HUVECs for any given treatment. Figure 5.9 shows the monocyte adhesion as a percentage of the value obtained from the TNF-α stimulated cells.

Incubating HUVECs with either 1mg/mL orosomucoid significantly decreased the adhesion of U937 monocytes to these cells. Monocyte adhesion to endothelial cells is the

important initial step in their migration through the endothelium and into the vascular interstitium, where they form lesions that eventually become atherosclerotic plaques (Lusis, 2000). Several adipokines are known to be involved in the various stages of formation of atherosclerotic lesions (see 5.3.1). This finding that orosomucoid is acting as an anti-inflammatory agent on endothelial cells, coupled with the fact that it is found in increased levels in the serum of fatty Zucker rats, suggests a role for orosomucoid as an anti-inflammatory adipokine.

5.5 Discussion

Of the 67 probable secreted proteins in Table 5.1, 39% are ECM proteins. This is a higher percentage than that found by Chen and colleagues (Chen et al., 2005). They suggest that the secreted proteome from 3T3-L1 adipocytes may have different characteristics from the primary adipocytes used in their study, on account of the physiological changes that 3T3-L1 cells have undergone by adapting to the cultured environment and that the extracellular matrix proteins secreted by 3T3-L1 adipocytes are stimulated in the culture of these cells. The protocol used by Chen and co-workers involves a collagenase digestion step which will remove collagens and severely disrupt the extracellular matrix. All adipose tissue *in vivo* secretes and is supported by an extra-cellular matrix, and therefore by including the extracellular matrix proteins in the proteome we see a more representative set of secreted proteins.

A number of proteins were identified in this study as potential new adipokines, several of which exhibited effects in functional assays to measure their effects on; insulin sensitivity in 3T3-L1 adipocytes; differentiation from pre-adipocytes to adipocytes and adhesion of U937 promonocytic cells to Human Umbilical Vein Endothelial cells

Orosomucoid exhibited anti-inflammatory properties by decreasing monocyte adhesion to human vascular endothelial cells. It also significantly inhibited insulin-stimulated glucose uptake. Orosomucoid has previously been shown to have anti-inflammatory or protective effects against inflammation (Kuzuhara et al., 2006; Hochepied et al., 2003). It strongly inhibits platelet aggregation (Costello et al., 1979) and more specifically, has been shown to interact directly with lipopolysaccharide (LPS), but the mechanisms by which it works are not known. It is a 41-43 kDa protein, which is composed of approximately 45% carbohydrate (five complex *N*-linked glycans)(Yoshima et al., 1981). Changes in these sugar residues appear early in the acute-phase of the inflammatory response, and IL-1, IL-6 and TNF-α appear to be involved in these modifications (van Dijk and Mackiewicz, 1995).

It has been suggested that the immunoprotective effects of orosomucoid are glycosylation dependent. Experiments have shown that desialylation of orosomucoid enhances its effect on platelet aggregation (Costello et al., 1979). Williams and co-workers have suggested that a sialyl Lewis X form of orosomucoid which is induced during inflammation, could mediate the immunomodulatory effects of the protein, since sialyl Lewis X is a ligand for the cell adhesion molecules E-selectin and P-selectin, both of which are involved in monocyte adhesion to endothelial cells (Williams et al., 1997).

Like Orosomucoid, Nm23 significantly reduced insulin-stimulated glucose uptake. The mechanisms by which it does this are unclear and further investigations o the actions of this protein will be necessary.

LRG1 and HSP90 appeared to have a small but statistically significant effect on Oil Red O uptake in differentiating 3T3-L1 adipocytes, suggesting that treatment with these recombinant proteins increased differentiation. Lysates made from cells treated with LRG1 and HSP90α during differentiation showed no obvious increase in expression of C/EBPα. Treatment with recombinant PF4 may cause an increase in expression of C/EBPα.

Neither of the secreted heat shock proteins, HSP70 nor HSP90α identified in this study had any effect in the functional assays, at the concentrations or times tested. They were both detected in the conditioned medium from 3T3-L1 adipocytes, and in the serum of lean and fatty Zucker rats, by Western blotting. Both of the heat-shock proteins appeared to be more abundant in fatty Zucker serum, than in lean, suggesting that these proteins are increased in obesity, and may play a role in the development of this condition. Previous studies have shown that extracellular HSP 70 and HSP90α have a pro-inflammatory effect on rat microglial cells, resulting in expression of cytokines, by activating NF-κB and p38-mitogen-activated protein kinase (MAPK), through the Toll-like receptor TLR4 (Kakimura et al., 2002). In this assay, cytokine production was seen after incubating migroglia for 24h with 10μg/mL of the HSPs. I saw no effect on endothelial cells treated for 4h with 5μg/mL. Further experiments are necessary to investigate the effects of different doses and incubation times of HSPs on endothelial cells.

The proteomic screen of proteins secreted by 3T3-L1 adipocytes has identified a range of proteins with the potential to be adipokines. Several of the proteins identified have been shown to influence the function of other cells. The anti-inflammatory effects of orosomucoid on HUVECs makes it a potential factors in atherosclerosis development. Orosomucoid, an acute-phase serum protein that has been found to be elevated in obesity

and Type II Diabetes, significantly decreased insulin-stimulated glucose uptake in 3T3-L1 adipocytes, making it a potential contributor to insulin resistance. These effects may be difficult to reconcile as they suggest that the same protein appears to ameliorate a condition associated with obesity, while appearing to worsen another. Until the mechanisms involved in these effects are elucidated, it is difficult to explain this anomaly. This question reinforces the need for further work to examine the biological activities and the mechanisms of action of these potential new adipokines.

Figure 5.1 SDS-PAGE of Proteins secreted from 3T3-L1 adipocytes

Figure 5.1 shows an SDS-PAGE representation of the proteins in the sample used for proteomic analysis

Figure 1

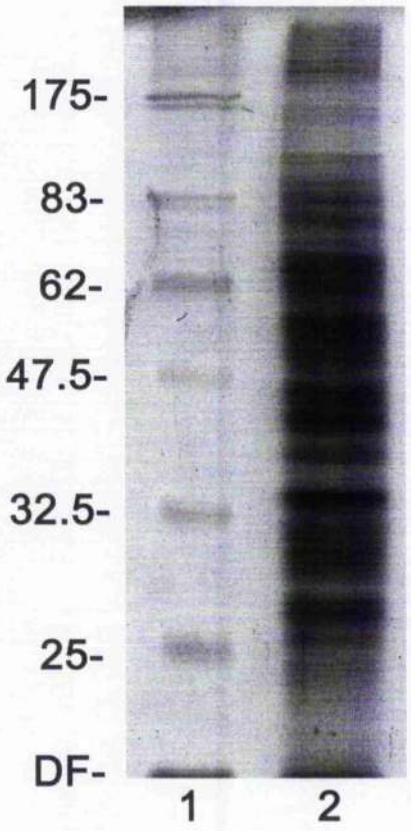


Figure 5.2
Mass Spectrum and Ions score data for manually validated protein

Figure 5.2a shows the mass spectrum for the manually validated protein hit; TSC-36/FRP (gi 2498391).

Figure 5.2b is the ions score table for TSC-36/FRP (gi 2498391).

a

320. gi|2498391 Score: 52 Queries matched: 2

Follistatin-related protein 1 precursor (Follistatin-like 1) (TGF-beta-inducible protein TSC-36)

☐ Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
2177	682.37	2044.07	2044.07	0.01	0	43	0.024	1	LIQWLEAEIIPDGWFSK 2

Proteins matching the same set of peptides:

gi|20892469 Score: 52 Queries matched: 2

b

{MATRIX SCIENCE} Mascot Search Results

Peptide View

MS/MS Fragmentation of **LIQWLEAEIIPDGWFSK**
Found in gi|2498391, Follistatin-related protein 1 precursor (Follistatin-like 1) (TGF-beta-inducible protein TSC-36)

Match to Query 2177: 2044.074264 from(682.365364,3+)

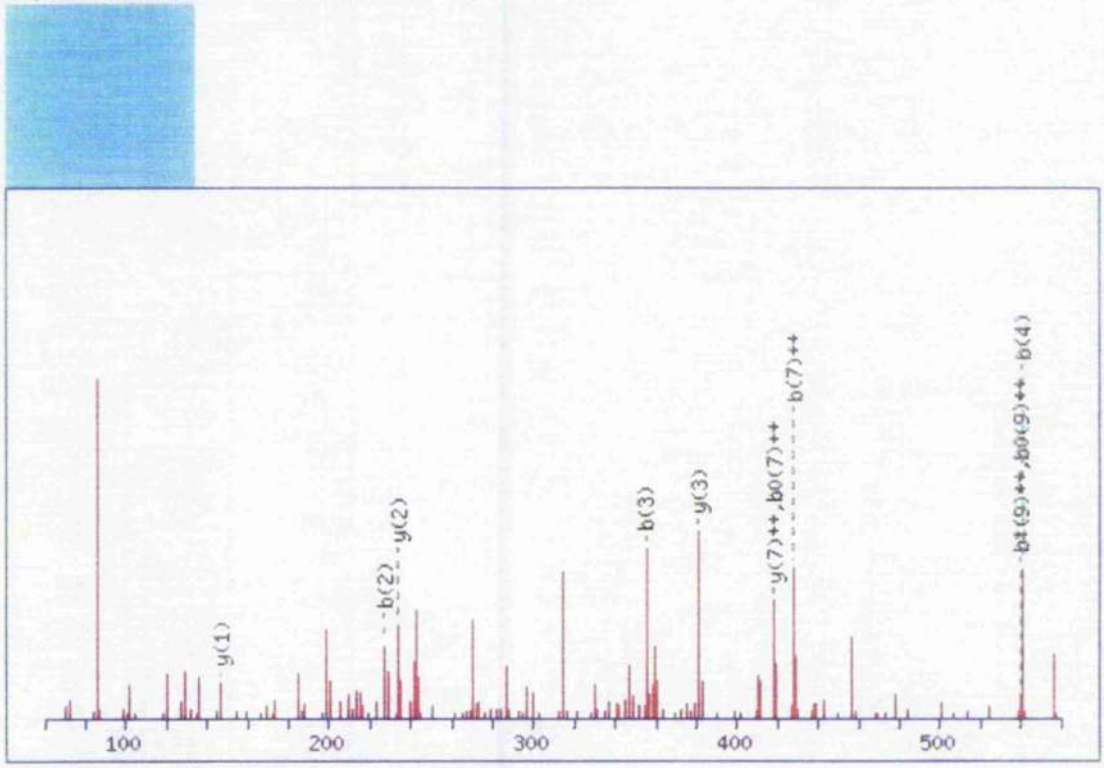
Sample: Washthrough Elution from: 65.79 to 65.79 period: 0 experiment: 2

cycles: 1

From data file C:\Analyst Data\Projects\Andy\Data\20_03_04MairiD.wiff

Click mouse within plot area to zoom in by factor of two about that point

Or, Plot from 160 to 440 Da



Monoisotopic mass of neutral peptide Mr(calc): 2044.07

Ions Score: 43 Expect: 0.024

Matches (Bold Red): 22/176 fragment ions using 47 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}
1	114.09	57.55					L						
2	**227.18**	114.09					I	1931.99	966.50	1914.96	957.99	1913.98	957.49

c

3	355.23	178.12	338.21	169.61			Q	1818.91	909.96	1801.88	901.44	1800.90	900.95
4	541.31	271.16	524.29	262.65			W	1690.85	845.93	1673.82	837.41	1672.84	836.92
5	654.40	327.70	637.37	319.19			L	1504.77	752.89	1487.74	744.37	1486.76	743.88
6	783.44	392.22	766.41	383.71	765.43	383.22	E	1391.68	696.35	1374.66	687.83	1373.67	687.34
7	854.48	427.74	837.45	419.23	836.47	418.74	A	1262.64	631.82	1245.61	623.31	1244.63	622.82
8	983.52	492.26	966.49	483.75	965.51	483.26	E	1191.60	596.31	1174.58	587.79	1173.59	587.30
9	1096.60	548.81	1079.58	540.29	1078.59	539.80	I	1062.56	531.78	1045.54	523.27	1044.55	522.78
10	1209.69	605.35	1192.66	596.83	1191.68	596.34	I	949.48	475.24	932.45	466.73	931.47	466.24
11	1306.74	653.87	1289.71	645.36	1288.73	644.87	P	836.39	418.70	819.37	410.19	818.38	409.70
12	1421.77	711.39	1404.74	702.87	1403.76	702.38	D	739.34	370.17	722.31	361.66	721.33	361.17
13	1478.79	739.90	1461.76	731.38	1460.78	730.89	G	624.31	312.66	607.29	304.15	606.30	303.66
14	1664.87	832.94	1647.84	824.42	1646.86	823.93	W	567.29	284.15	550.27	275.64	549.28	275.14
15	1811.94	906.47	1794.91	897.96	1793.93	897.47	F	381.21	191.11	364.19	182.60	363.20	182.10
16	1898.97	949.99	1881.94	941.47	1880.96	940.98	S	234.14	117.58	217.12	109.06	216.13	108.57
17							K	147.11	74.06	130.09	65.55		

NCBI BLAST search of LIQWLEAEIIPDGWFSK

(Parameters: blastp, nr protein database, expect=20000, no filter, PAM30)

Other BLAST web gateways

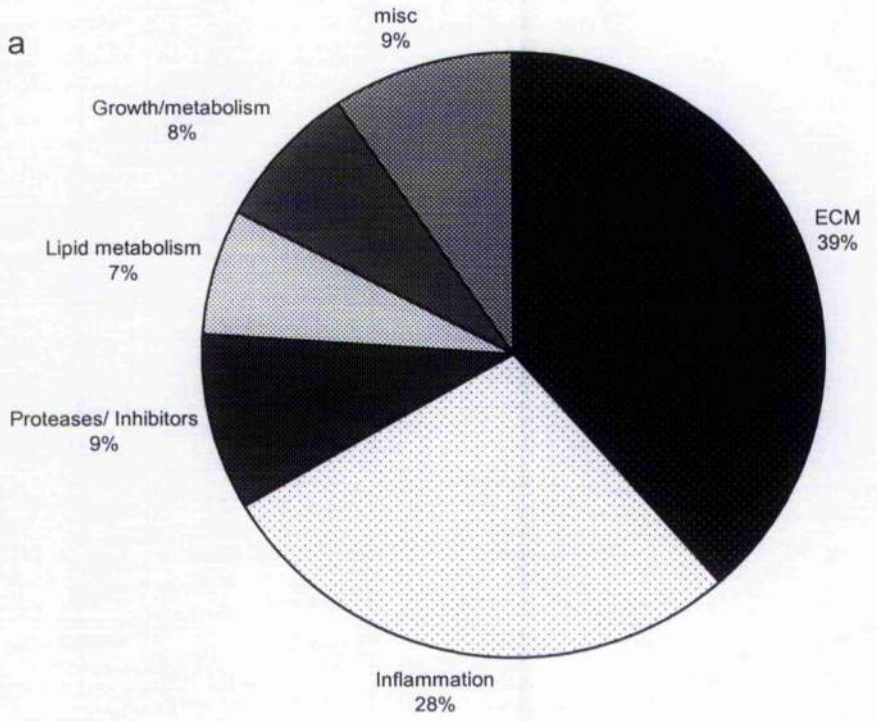
Mascot: http://www.matrixscience.com/

Figure 5.3 Distribution of secreted proteins by function

Pie-charts illustrating relative distribution of secreted proteins between this study and the study of Chen and co-workers (Chen et al., 2005)

Figure 5.3a shows the distribution, according to function, of proteins identified in this study

Figure 5.3b shows the distribution, according to function, of the the combined proteins identified in this study and in the study of Chen and co-workers.


a
misc
9%
Growth/metabolism
8%
Lipid metabolism
7%
Proteases/ Inhibitors
9%
ECM
39%
Inflammation
28%

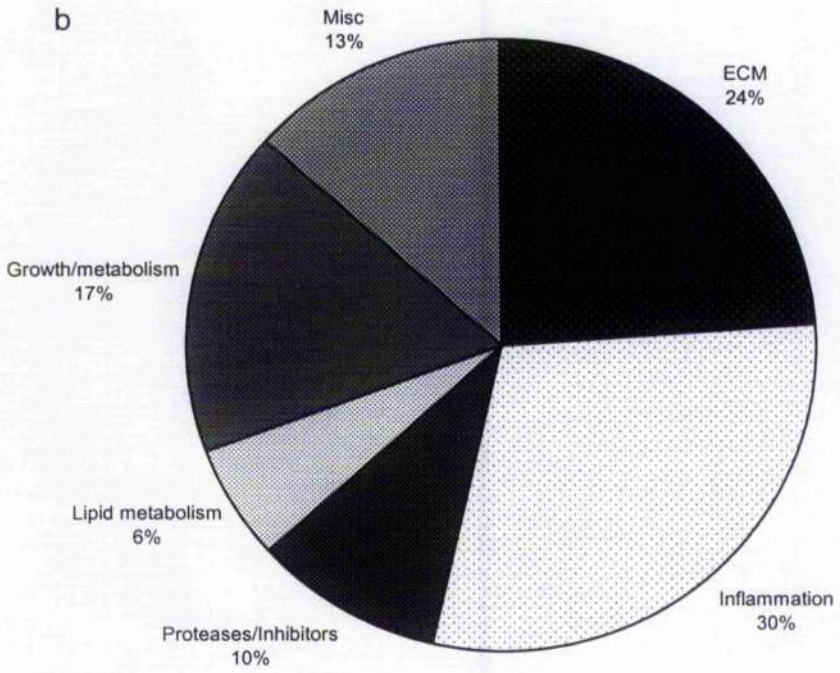

b
Misc
13%
ECM
24%
Growth/metabolism
17%
Lipid metabolism
6%
Proteases/Inhibitors
10%
Inflammation
30%

Figure 5.4 Secretion of HSP70, HSP90α and Orosomucoid by 3T3-L1 adipocytes

Figure 5.4 shows that HSP90α, HSP70 and orosomucoid are detectable by immunoblotting in the conditioned medium from 3T3-L1 adipocytes.

This is a single experiment n=1

a insulin - +

hsp90

b insulin - +

hsp70

c insulin

- +

orosomucoid

Figure 5.5
HSP90α and Orosomucoid in plasma from Lean and Fatty Zucker Rats

HSP90α, HSP70 and orosomucoid are detectable in plasma, by immunoblotting. HSP90α and orosomucoid are present in higher levels in the plasma of Zucker Fatty rats than in Zucker Lean rats. HSP70 is detectable in the plasma of Zucker Fatty rats, but not detectable by this method in the plasma of Zucker Lean rats.

To show that equal volumes of serum were loaded in each well, samples were blotted with ant-ratIgG.

Each blot shows the results from one experiment. n=1

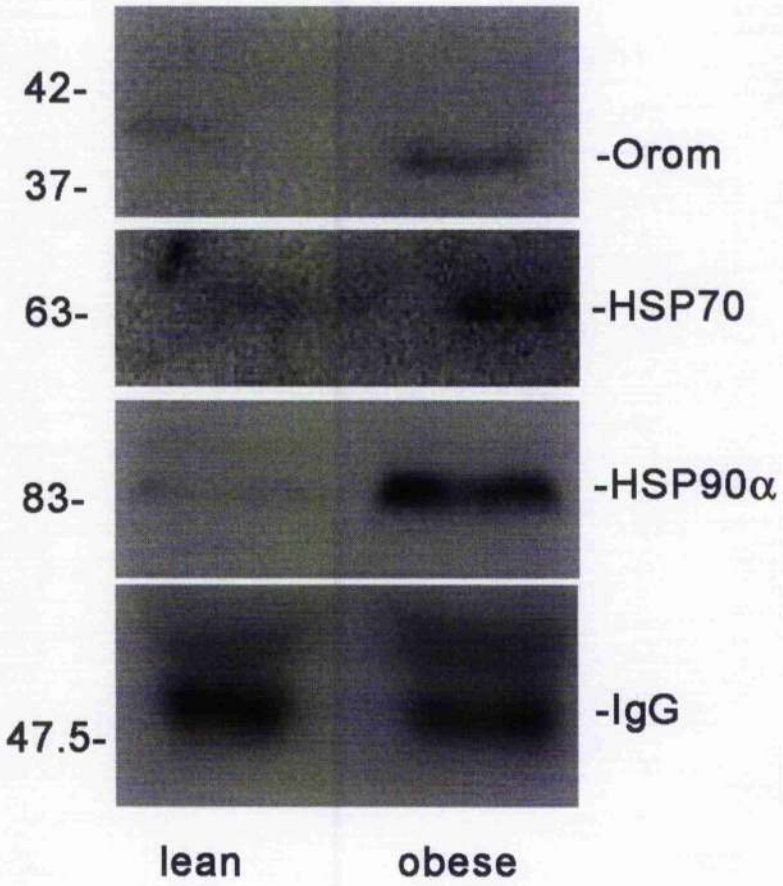

42-
37-
-Orom
63-
-HSP70
83-
-HSP90α
47.5-
-IgG
lean
obese

Figure 5.6 Effect of recombinant proteins on Insulin-Stimulated 2-Deoxy-D-Glucose uptake in 3T3-L1 adipocytes

Pre-incubation of 3T3-L1 adipocytes with 10μg/mL or 30μg/mL orosomucoid resulted in a significant reduction in insulin-stimulated glucose uptake by 3T3-L1 adipocytes.

Pre-incubation with Nm23 resulted in a small but statistically significant reduction.

The results from each condition are expressed as a percentage of the control sample (no protein treatment, stimulation with 100nM insulin).

* Indicates a result with a value of $p \leq 0.05$.

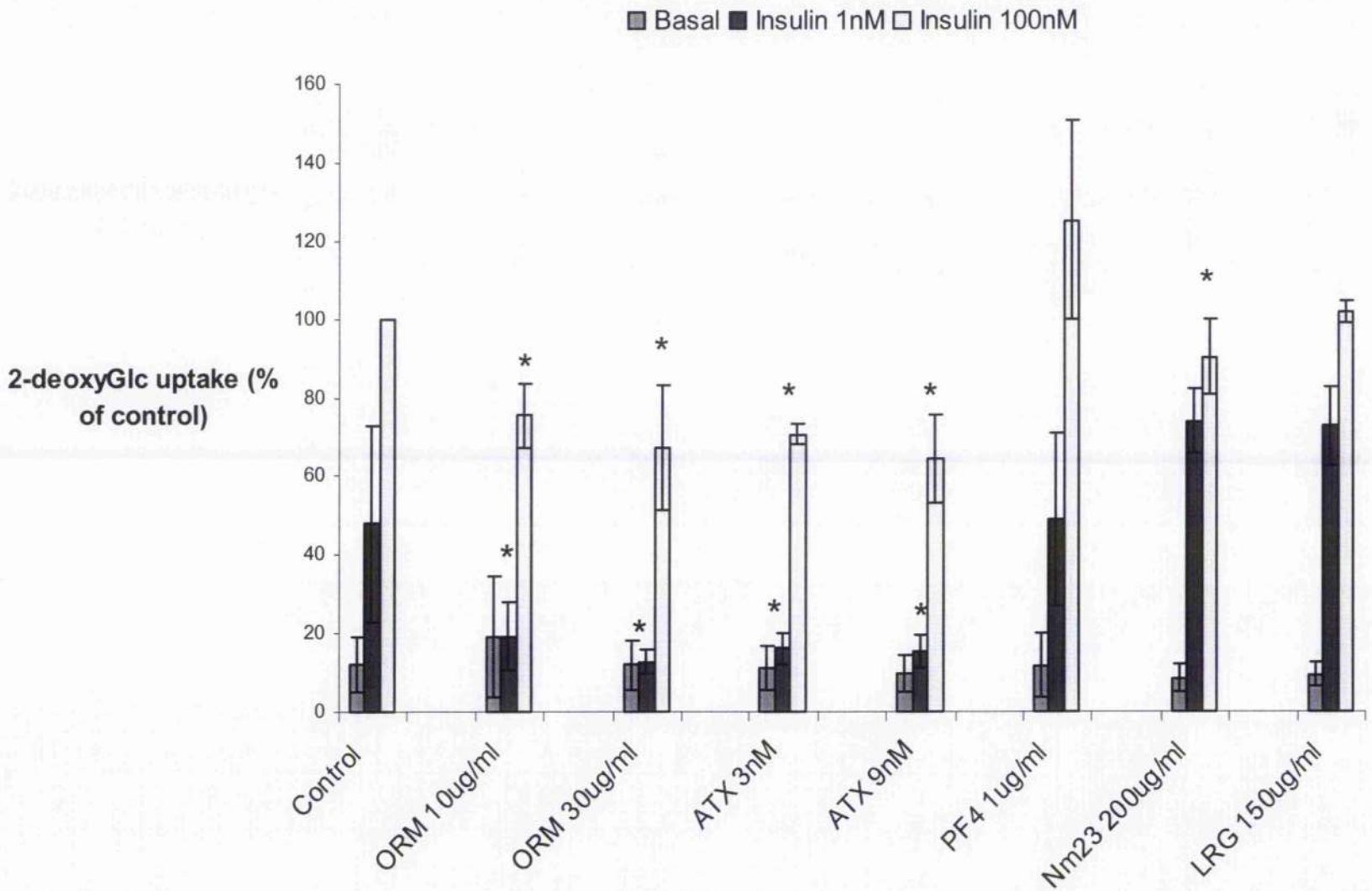

Basal
Insulin 1nM
Insulin 100nM
2-deoxyGlc uptake (% of control)
160
140
120
100
80
60
40
20
0
Control
ORM 10ug/ml
ORM 30ug/ml
ATX 3nM
ATX 9nM
PF4 1ug/ml
Nm23 200ug/ml
LRG 150ug/ml

Figure 5.7 Oil Red O Uptake by 3T3-L1 adipocytes treated with recombinant proteins

Incubation of 3T3-L1 adipocytes with 5μg/mL HSP90α or with 75ng/mL LRG1 during differentiation of pre-adipocytes, resulted in a small but statisically significant increase in uptake of Oil Red O.

The results are expressed as OD_{520}

The results shown are the combined results of n =3 for all recombinant protein, except HSP70 (n=2).

* Indicates a result with a value of $p \leq 0.05$.

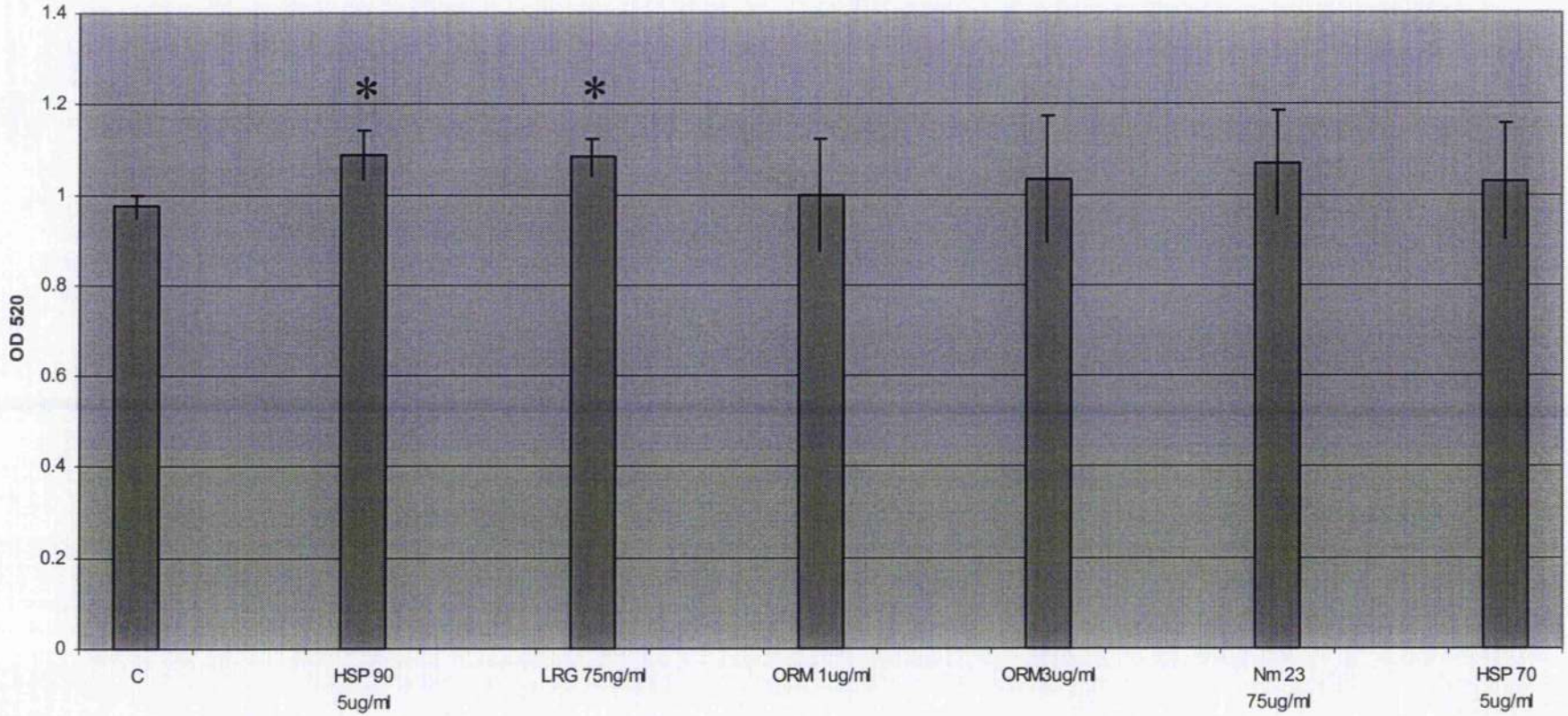

1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 520
*
*
C
HSP 90
5ug/ml
LRG 75ng/ml
ORM 1ug/ml
ORM3ug/ml
Nm 23
75ug/ml
HSP 70
5ug/ml

Figure 5.8 Expression of the transcription factors CEBPα and PPARγ in 3T3-L1 adipocytes treated with recombinant proteins

Incubating 3T3-L1 adipocytes with PF4, orosomucoid, Nm23, LRG1, HSP90α and HSP70 had no effect on expression of the differentiation associated transcription factor PPAR-γ. There appears to be an increase in the expression of C/EBPα with PF4-treated 3T3-L1 adipocytes. These blots shown are from a single experiment, in which at least two wells were combined to make each lysate.

Equal loading was determined by immunoblotting for GAPDH.

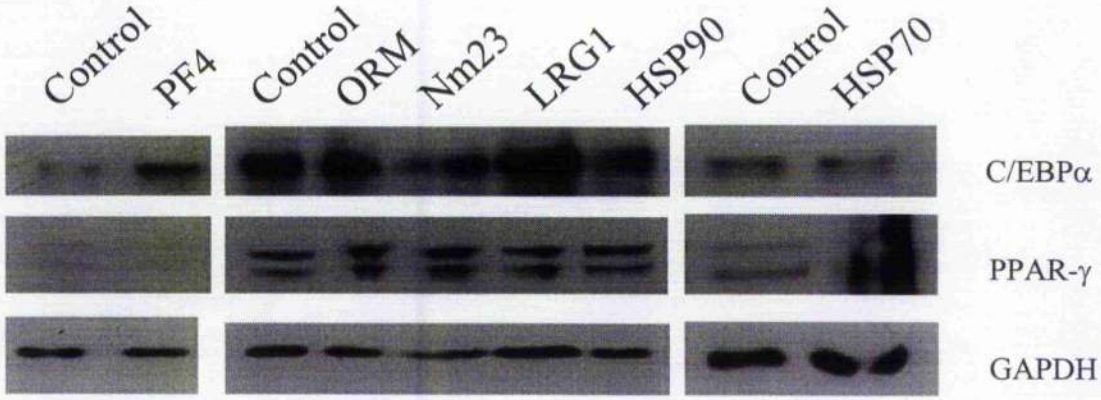

Control
PF4
Control
ORM
Nm23
LRG1
HSP90
Control
HSP70
C/EBPα
PPAR-γ
GAPDH

Figure 5.9 Adhesion of U937 monocytes to HUVECs treated with recombinant proteins

Human Umbilical Vein Endothelial Cells incubated with 1mg/mL orosomucoid, resulted in a significant decrease in adhesion of U937 cells.

This shows an n =3. Error bars show standard deviation

^ Indicates a result with a value of $p \leq 0.01$

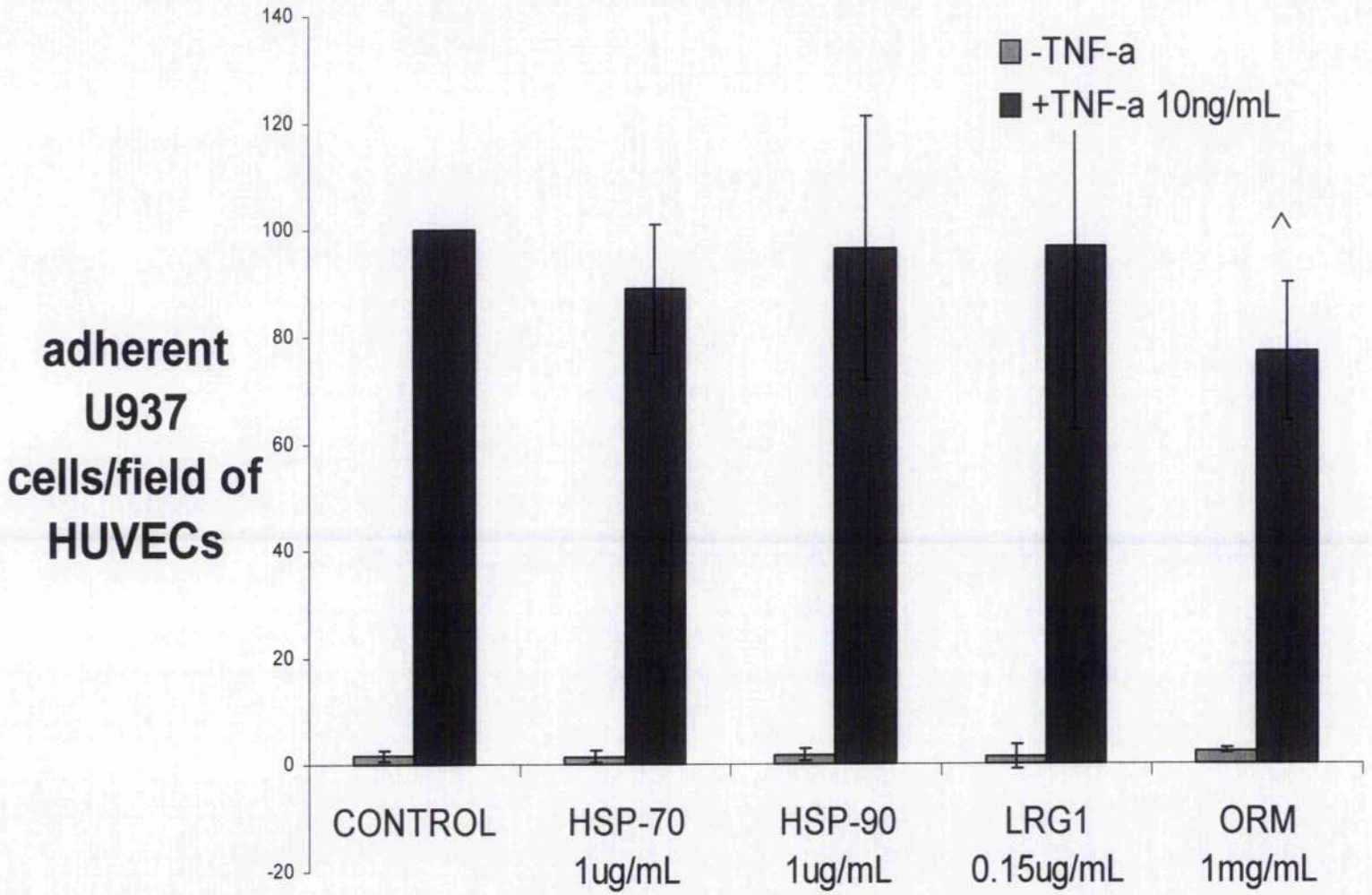

adherent U937 cells/field of HUVECs
140
120
100
80
60
40
20
0
-20
-TNF-a
+TNF-a 10ng/mL
^
CONTROL
HSP-70 1ug/mL
HSP-90 1ug/mL
LRG1 0.15ug/mL
ORM 1mg/mL

Table 5.1 3T3-L1 adipocyte-secreted proteins identified by proteomic analysis

Table 5.1

Protein	Score	Function	Database Accession Number
PEDF	173	Serpin	gi 2961472
Serine protease inhibitor	75	Serpin	gi 27806487
Unnamed Prot product	326	Trypsin Inhib?	gi 12836422
Adipsin	172	Ser. protease	gi 673431
Complement C1r	149	Ser.protease/Inflamm	gi 12963529
Complement C1s	82	Ser.protease	gi 21450097
Complement C1q	159	Ser.protease	gi 6137745
Complement C3	1513	Inflammation	gi 28175786
Complement C3d	101	Inflammation	gi 9954969
Complement C4	817	Inflammation	gi 2944420
Angiotensinogen	853	Serpin	gi 113881
MIF	100	Pro-inflammatory cytokine	gi 694108
ACRP30	121	Inhibitor of Inflammation	gi 21426809
Haptoglobin	620	Acute phase protein	gi 2144486
ceruloplasmin	201	Ferroxidase	gi 6680997
Extracellular SOD	229	Antioxidant/	gi 3320907
Periostin	107	Activin binding	gi 7657429
Lipoprotein lipase	426	Lipid metabolism	gi 387406
Lipid binding protein	1726	Lipid binding	gi 3318748
PE binding protein	199	Lipid binding	gi 12841975
IGF BP 7	395	Binds IGF	gi 28502909
CyC C-AP	348	SCY chemokine	gi 397800
Cyclophilin A	89	Chemotaxis	gi 1431788
Nucleobindin	122	Ca binding	gi 6679158
Platelet factor 4/ CXCL4	84	SCY cytokine/chemotactic	gi 266800
Thrombospondin2	312	Adhesive glycoprotein	gi 6755779
Orosomucoid	273	Acute phase protein	gi 15215270
Selenium binding protein	117	Se/lipid binding	gi 134259 (NCS)
Sex limited protein	491	Acute-phase	gi 220604
Collagen VI α1	657	ECM	gi 6753484
Collagen VI α2	328	ECM	gi 3913189
Collagen VI α3	1493	ECM	gi 31791061
Vimentin	139	IF	gi 5030431
Fibronectin	193	ECM	gi 38372875
Laminin B1	206	ECM	gi 126367
GelatinaseA/MMP2	69	Regulation of ECM	gi 1813503
ECM associated protein	154	SPARC-like	gi 1498641
Osteonectin	146	SPARC/ HSP45	gi 600381
Autotaxin-t	392	Monocyte chemotaxis 5'-nucleotide PDE activity	gi 1160616
Gelsolin	227	Actin binding	gi 2833344
HSP 90α	102	MMP2 activation	gi 41200885

Protein	Score	Function	Database Accession Number
HSP 70	377	Secreted HSP/cytokine	gi 72288
Fibulin-2	174	ECM	gi 4959705
Similar to Collagen XV	65	ECM	gi 34868476
Perlecan	92	ECM	gi 6680311
Versican	54	ECM	gi 21361116
Vitronectin	73	ECM	gi 73587073
Lamnin α4	401	ECM	gi 1869967
Decorin	151	Sm. proteoglycan	gi 6681143
Biglycan	499	Sm. proteoglycan	gi 348962
Leu.richα-2 glycoprotein	88	secreted glycoprotein	gi 16418335
NDPK B	84	?same as I factor	gi 6679078

Manually Validated confident			
α-amylase	67	Digestive enzyme	gi 67370
Thioredoxin	50	Cytokine	gi 16740761(NCS)
ApolipoproteinA-I bp	67	Lipid binding	gi 21553309
CyP-S1	60	? Chemotactic	gi 2118329
Cathepsin B	52	Cys. Protease(Lysosomal)	gi 227293
Laminin B2	67	ECM	gi 293691
Unnamed protein product	56	Likely leucine aminopeptidase	gi 195269(NCS)
TSC-36/FRP	52	Inhibition of growth/diffn	gi 2498391
Nm23/I Factor	67	Inhibition of differentiation	gi 35068
Leucine aminopeptidase	57	M1 secreted peptidase	gi 13161142
Serpin 3n	81	Serpin	gi 75765653

MV not confident			
Thrombospondin 1	45	Adhesive glycoprotein	gi 567240
PBEF/visfatin	43	Cytokine	gi 10946948
MKIAA02320/peroxidasin	52	ECM stability	gi 28972103

Table 5.2 Common proteins identified by this study and by Chen and co-workers

Table 5.2

Protein	Database accesssion number
PEDF	gi 2961472
Adipsin	gi 673431
Complement C1	gi 12963529
Complement C4	gi 2944420
Angiotensinogen	gi 113881
MIF	gi 694108
ACRP30	gi 21426809
Haptoglobin	gi 2144486
Lipoprotein lipase	gi 387406
IGF bp7	gi 28502909
Cyclophilin A	gi 1160616
Nidogen	gi 6754854
Gelatinase A/MMP2	gi 1813503
Collagen IV α 1	gi 34879634
Vimentin	gi 5030431
Laminin B	gi 126367
Nucleobindin	gi 189308
Cathepsin B	gi 227293
Gelsolin	gi 2833344
Thioredoxin	gi 16740761

Table 5.3 Extracellular Matrix Proteins identified in both this study and that of Chen and co-workers

Table 5.3

Protein	Functon	Database Accession number
*Procollagen V α 2	ECM	gi 6680970
*Laminin B	ECM	gi 817975
**ADAMTS-17	Matrix metalloproteinase	gi 37999850
*MMP 2	ECM regualtion	gi 13591991
**MMP 9	ECM regualtion	gi 13591993
**TIMP-1	ECM regulation	gi 1174697
*nidogen	ECM interaction	gi 6754854
*osteonectin	SPARC	gi 600381
**Laminin γ-1 chain	ECM	gi 20825636
**tetranectin	ECM	gi 27721871
Thrombospondin 2	Adhesive glycoprotein	gi 554390
MKIAA02320/peroxidasin	ECM stability	gi 28972103
Fibulin-2	ECM	gi 4959705
ProcollagenC proteinase enhancer	ECM associated	gi 6919942
Collagen I α1	ECM	gi 2506305
Collagen I α2	ECM	gi 16758080
Collagen II α1	ECM	gi 109679
Collagen III α1	ECM	gi 2119163
*Similar collagen IV α1	ECM	gi 34879634
Collagen VI α1	ECM	gi 6753484
Collagen VI α2	ECM	gi 3913189
Collagen VI α3	ECM	gi 31791061
Collagen VII α 1	ECM	gi 6680972
Similar to Collagen XV	ECM	gi 34868476
Fibronectin	ECM	gi 38372875
Perlecan	ECM	gi 6680311
Papilin	ECM proteoglycan	gi 18700030
Versican	ECM	gi 21361116
Vitronectin	ECM	gi 36573
*Laminin B	ECM	gi 126367
Lamnin α4	ECM	gi 1869967
Decorin	Sm. proteoglycan	gi 6681143
Biglycan	Sm.proteoglycan	gi 348962
ECM associated protein	SPARC-like	gi 1498641
papilin	ECM	gi 18700030
Similar tenascin R	ECM/Inhibition of diffn	gi 28483364

*Clarke+ Chen **Chen

Table 5.4 Potential novel adipokines identified in this study

Table 5.4

Novel secreted proteins

Protein	score	function	Accession number
Unnamed Prot product	326	Trypsin Inhib?	gi 12836422
CyC C-AP	348	SCY chemokine	gi 397800
Platelet factor 4/ CXCL4	84	SCY cytokine/chemotactic	gi 266800
Thrombospondin2	312	Adhesive glycoprotein	gi 6755779
Orosomucoid	273	Acute phase protein	gi 15215270
Selenium binding protein	117	Se/lipid binding	gi 134259 (NCS)
Sex limited protein	491	Acute-phase	gi 220604
ECM associated protein	154	SPARC-like	gi 1498641
HSP 90α	102	MMP2 activation	gi 41200885
HSP 70	377	Secreted HSP/cytokine	gi 72288
Fibulin-2	174	ECM	gi 4959705
Similar to Collagen XV	65	ECM	gi 34868476
Perlecan	92	ECM	gi 6680311
Versican	54	ECM	gi 21361116
Vitronectin	73	ECM	gi 73587073
Laminin α4	401	ECM	gi 1869967
Decorin	151	Sm. proteoglycan	gi 6681143
Biglycan	499	Sm. proteoglycan	gi 348962
NDPK B	84	?same as I factor	gi 6679078
α-amylase	67	Digestive enzyme	gi 67370
CyP-S1	60	? Chemotactic	gi 2118329
TSC-36/FRP	52	Inhibition of growth/diffn	gi 2498391
Nm23/I Factor	67	Inhibition of diffn	gi 35068 to
Serpin 3n	81	Serpin	gi 75765653
Leu. richα-2 glycoprotein	88	Secreted glycoprotein	gi 16418335

Chapter 6 Summary and Perspectives

6.1 Secretion of ACRP30 in 3T3-L1 adipocytes

Adipocytes secrete a variety of proteins that are involved in metabolism, the immune response and cardiovascular function. Several of these adipokines have been studied and well characterised, including adipsin, TNF-α, leptin and ACRP30. Relatively little however, is known about the trafficking of these adipokines or about the mechanisms involved in their secretion. Unlike secretory neuroendocrine cells, there is no evidence for regulated secretion in adipocytes, other than the insulin-responsive trafficking of GLUT4 to the cell surface. No other secreted protein has been found sequestered in secretory vesicles, awaiting the stimulus that sends it to the extracellular environment, rather there appear to be many pathways of secretory traffic to the cell surface in secretory cells (see Introduction Section 1.5.1). Insulin-stimulation of 3T3-L1 adipocytes causes only a modest increase in ACRP30 secretion (Figure 3.4), more in common with recycling of the transferrin receptor than trafficking of GLUT4. In specialised secretory cells, like neuroendocrine cells, Arvan *et al.* observed that in addition to secretion directly from the TGN to the PM, there is traffic of secretory cargo from the TGN to ISGs and from there to the PM via the endosomal system. This led them to suggest that constitutive-like secretion involves two limbs: one from ISGs to endosomes and the other from endosomes to the PM.

By examining the intracellular distribution (Figure 3.5), and by disrupting trafficking through the TGN, by treatment with Brefeldin A (Figure 3.6) and trafficking through the endosomes by endosomal ablation(Figure 3.7), I have shown clear evidence that the adipokine ACRP30 is secreted in a constitutive-like pathway, in vesicles that traffic to the PM via the TGN and endosomes. This result parallels those from previous investigations of the secretory routes taken by other adipokines including leptin (Bradley et al., 2001) and adipsin (Yang and Mueckler, 1999).

I have also demonstrated roles for the small GTPases Rab11 and Arf6 in the trafficking of ACRP30 to the PM in 3T3-L1 adipocytes. Rab11 is a small GTPase that controls vesicular trafficking between the endosomes and the PM. Overexpression of the GDP-restricted Rab11-S25N mutant, reduced basal and insulin-stimulated secretion of ACRP30, provides evidence that ACRP30 traffics via endosomes, on its way to the PM.

Overexpression of GTP or GDP-restricted mutants of small molecular weight GTPases in mammalian cells is a very useful tool for dissecting intracellular membrane trafficking. As

a method it has been responsible for giving insight into the role of the small GTPases throughout the cell (Ullrich et al., 1996; Wilson et al., 1994; Chavrier and Goud, 1999; Martinez et al., 1997). Infecting adipocytes with adenoviruses expressing these small GTPases can be problematic. Adipocytes are not as easy to infect as mammalian fibroblasts such as HeLa or CHO cells, and therefore much higher virus titers are needed for successful infection. There are however alternative approaches for looking at the role of Arf and Rab proteins in the secretory pathway in 3T3-L1 adipocytes. The method of knocking down protein expression by small interfering RNAs (siRNA) has been used successfully in adipocytes (Mitra et al., 2004) and could be used in future experiments to further elucidate the role of Rab11 and Arf6 in the secretory process in 3T3-L1 adipocytes. By knocking down Rab11 or Arf6, we could examine the effects on secretion of ACRP30. There is evidence that some small GTPases work in close association with others. An example of this is Rab11/Rab25. Using siRNA to knock down both of these Rabs might give a clearer picture of how they are invovlved in ACRP30 secretion and GLUT4 trafficking. I saw no effect on ACRP30 secretion by overexpressing Arf5 wt and Arf5T31N. Arf 5 is also known to work closely with Arf4, and other groups have shown tha tit is necessary to knock down Arfs 4 and 5 together to see a morphological effect within the cell. Using si RNA to knock down Arf4 and Arf5 in 3T3-L1 adipocytes may reveal a role for these in the trafficking of secretory molecules in adipocytes.

Several Rab11 effector molecules have been identified, belonging to the Rab11 family of interacting proteins (FIP), that appear to have distinct roles in regulating membrane traffic between the recycling endosomes and the PM (Prekeris, 2003). The role of Rab11 effector molecules in recycling is an area that could be investigated in 3T3-L1 adipocytes, by using techniques such as expression knockdown by si RNAs and immunofluorescence.

As more adipokines are identified, it becomes more important to understand the mechanisms controlling their secretion. As obesity progresses, adipocytes become larger and display an altered pattern of secretion. Some adipokines exhibit increased secretion and others are reduced. This study has revealed information about the pathway taken by adiponectin from its synthesis to its secretion, and the role of certain GTPases in regulating that secretion. Very little is yet known about the control of secretion of the various adipokines and why there is a difference in the expression of adipokines from anti-inflammatory to pro-inflammatory with progression of obesity and increase in adipocyte size. Using some of the techniques employed in this study, it will be possible to investigate the mechanisms of secretion of other adipokines, including several of those potential new adipokines identified in this work, and by illustrating the molecules and mechanisms that

are involved in secretion, so identify potential pharmacological targets in the adipocyte secretory pathway.

6.2 Secreted proteins – novel adipokines?

In a detailed proteomic screen of the secreted proteins of mature day 10 3T3-L1 adipocytes, a number of proteins were identified as potential new adipokines with several of them exhibiting effects in functional assays. The screen has identified two secreted proteins, not previously been identified as being secreted by adipocytes, orosomucoid and Nm23 that significantly affect insulin-stimulated glucose uptake. In addition, orosomucoid exhibited anti-inflammatory properties by decreasing monocyte adhesion to human vascular endothelial cells. LRG1 and HSP90 appeared to have a small but statistically significant effect on Oil Red O uptake in differentiating 3T3-L1 adipocytes, suggesting that treatment with these recombinant proteins increased differentiation. Lysates made from cells treated with LRG1 and HSP90α during differentiation showed no obvious increase in expression of C/EBPα. Treatment with recombinant PF4 may cause an increase in expression of C/EBPα.

A large number of the adipocyte-secreted proteins identified are ECM or ECM associated proteins and I have identified several ECM and ECM associated proteins that have not previously been identified as adipocyte secreted proteins. These include collagens and the adhesive proteins that make up the basal lamina, and the small leucine-rich proteoglycans, decorin and biglycan. A picture emerges from the list of ECM and ECM associated proteins of a complex environment surrounding the cells. Adipocytes secrete matrix proteins like collagen and laminin and adhesive proteins in order that they can attach to the matrix. They also secrete proteases and metalloproteinases that modify the matrix, allowing for cell movement and the release of growth factors. Finally, they secrete protease inhibitors, which control the action of the proteases. The regulation of secretion of each of these proteins is vital during the development and normal function of healthy tissue, and a loss of this balance has been associated with various states of disease: Matrix metalloproteinases (MMPs) are endoproteinases that are involved in ECM remodelling. Extracellular HSP90α is involved in the maturation of MMP2, and through this action promotes the invasion of cancer cells through the ECM (Eustace et al., 2004b). It is possible that HSP90α secreted by fat cells assists the remodelling of adipose tissue in the same way. Metalloproteinases and their inhibitors have been implicated as factors in tumour cell migration, angiogenesis and atherosclerosis Reviewed in (Bornstein and Sage,

2002). Evidence suggests that the ECM, through its interaction with cytokines and proteases, provides signals for the recruitment of monocytes during their recruitment to sites of inflammation (Vaday and Lider, 2000). The development of adipose tissue depends not only upon the development of a supporting matrix but also on the means to remodel that matrix, since as fat tissue mass increases, so does the requirement for blood vessel development, and a vital step of angiogenesis is the degradation of connective tissue by secreted proteases. A possible future direction for study in this area might be to search, using a process called positional proteomics that relies on the recovery and analysis of N-terminal proteolytic peptides (McDonald et al., 2005), for peptides that have been generated by proteolysis as they are secreted from the cell.

This might help to build a picture of the role of the proteases that are secreted and might reveal more about how they are involved in remodelling of the extracellular environment. Obesity is also marked by infiltration of fat tissue by macrophages, a process that also requires matrix remodelling. It has been suggested that angiogenesis could be a target for future therapy and that inhibitors of angiogenesis and matrix protease inhibitors might be employed as anti-obesity therapy

Several of the novel secreted proteins identified are known to be involved in inflammation, either as acute-phase proteins or pro-inflammatory cytokines. Obesity is characterised by a state of inflammation and an accumulation of macrophages in adipose tissue, and these macrophages contribute to the insulin resistant state that is associated with obesity and type 2 diabetes (Xu et al., 2003). With inflammation playing such a strong role in the development of diabetes, these adipocyte-secreted inflammatory proteins may help to elucidate the mechanisms that link obesity, insulin resistance and diabetes.

Orosomucoid exhibited anti-inflammatory effects on endothelial cells, resulting in reduced adhesion of U937 cells. There are reports of the anti-inflammatory effects of orosomucoid in various assays but the mechanisms by which it elicits these effects are unclear. Results from other laboratories suggest that the anti-inflammatory effect is dependent on the glycosylation of the protein and that its carbohydrate chains may be ligands for cell adhesion molecules and therefore compete with the ligands on the U937 cells. If this is the reason for the anti-inflammatory effects of orosomucoid, then it should be possible to observe an effect without having to pre-incubate endothelial cells with the protein for 4h. TNF-α stimulates monocyte adhesion to endothelial cells by activation of the IκB/NFκB signalling cascade, resulting in increased expression of cell surface adhesion molecules. Future experiments might test the hypothesis that orosomucoid interferes with this

signalling. This could be done by assaying NFκB-mediated transcriptional activation. Another approach might be to determine, by flow cytometry, whether orosomucoid affects the expression of cell surface adhesion molecules such as VCAM-1 and ICAM-1. The anti-inflammatory effect was seen using orosomucoid at 1mg/mL and under inflammatory conditions orosomucoid is found circulating in serum at concentrations up to 3mg/mL. Future experiments should measure any dose-dependent effects. As orosomucoid is a potential anti-inflammatory adipokine, future studies might involve investigating its therapeutic possibilities by administering it to animal models of diabetes and obesity.

The heat shock proteins HSP70 and HSP90α, which were both found to be secreted by 3T3-L1 adipocytes, had no significant effects in the functional assays, at the concentrations tested. Further experiments are required to establish the role of these extracellular heat shock proteins. HSP 70, HSP90 and orosomucoid were all identified, by Western blotting, in the serum from lean and fatty Zucker rats and appeared to circulate in higher concentrations in fatty serum. This increase is suggestive of a role for these proteins in obesity and future work should involve an examination of the levels of these adipokines in human populations. A determining factor of adipokines is that their serum levels correlate with factors associated with insulin resistance or type 2 diabetes and therefore it will be important to determine whether serum levels correlate with obesity or insulin resistance.

All of the proteins that have exhibited an effect in these functional assays should be studied further. It will be important to investigate the mechanisms, by which they are acting, to find out how, as extracellular proteins, they bring about intracellular changes. What are the signalling pathways that are involved? How do the serum levels of these secreted proteins in normal individuals compare with that of patients with diseases such as obesity and Type II diabetes? These are all possible future directions for this project.

Publications arising from the work in this thesis:

Syntaxin6 regulates GLUT4 trafficking in 3T3-L1 adipocytes (Perera et al., 2003),

Evidence of a role for the exocyst in insulin-stimulated GLUT4 trafficking in 3T3-L1 adipocytes (Ewart et al., 2005),

ACRP30 is secreted from 3T3-L1 adipocytes via a Rab11-dependent pathway(Clarke et al., 2006)

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