STUDIES OF THE TRAFFICKING OF THE INSULIN-RESPONSIVE GLUCOSE TRANSPORTER, GLUT4, IN 3T3-L1 ADIPOCYTES

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Declaration

I declare that the work described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree

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ABSTRACT

The translocation of GLUT4 from intracellular stores to the plasma membrane in response to insulin accounts for the large insulin-mediated glucose uptake in muscle and fat tissue. A defect of this translocation mechanism is evident in individuals with insulin resistance and type 2 diabetes. Hence, understanding the molecular basis of GLUT4 localisation and recycling is important in order to assist the design of rational therapies for the treatment of this disease.

Here, we have used iodixanol gradient analysis to examine the intracellular distribution of GLUT4. By this method intracellular GLUT4 could be separated into two pools one of which is highly insulin sensitive, and corresponds to "GLUT4 storage vesicles" (GSV), while the other is less insulin sensitive and is of endosomal origin. We further show that during differentiation of 3T3-L1 fibroblasts into adipocytes, the formation of the GSV compartment appears to be driven by the expression of GLUT4.

SNARE proteins are involved in the fidelity of GLUT4 translocation, but little is known about how these proteins may be altered in insulin resistance. Using insulin-resistant 3T3-L1 adipocytes, we show that SNARE protein levels are increased. The potential importance of this observation is discussed. Using iodixanol gradient analysis we also found that the downregulation of GLUT4 known to occur in insulin-resistant 3T3-L1 adipocytes is predominantly from the GSV compartment. The implications of these data for the aetiology of insulin resistance are discussed.

Finally we show that the adaptor protein distribution in 3T3-L1 adipocytes is not affected by insulin. Furthermore, we demonstrate that GLUT4 as well as IRAP can interact with some of the adaptors known to be important in trafficking at the TGN and endosomes. This data therefore supports a role of these adaptor proteins in GLUT4 recycling.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 5

ABBREVIATIONS

Ade	Adenine
AP	Adaptor protein
ARF	Adenosine diphosphate ribosylation factor
ATB-BMPA	2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis (D-mannos-4-yloxy)-2-propylamine
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
cpm	counts per minute
CPRG	Chlorophenol red-β-D-galactopyranoside
CSP	Cysteine string protein
DAB	3.3' Diaminobenzidene
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDAC	N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Diaminoethanetetra-acetic acid, Disodium salt
EM	Electron microscopy
EGFP	Epidermal growth factor receptor
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
gal	Galactosidase
GLUT	Glucose transporter
GTP	Guanosine 5'-triphosphate
GTPγS	Guanosine 5'-(γ-thio) triphosphate
GSV	GLUT4 storage vesicle
h	Hours
HDM	High density microsome/membrane
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HES	HEPES EDTA Sucrose
His	Histidine
HRP	Horseradish peroxidase
IBMX	Methyl isobutylxanthine
IgG	Immunoglobulin gamma
IP	Immunoprecipitation
IRAP	Insulin responsive amino peptidase
IRS	Insulin receptor substrate
KRP	Krebs Ringer Phosphate
KRMES	Krebs Ringer MES
LDM	Low density microsome/membrane
Leu	Leucine
LiAc	Lithium acetate
Lys	Lysine
mA	Milliamps
MES	2-(N-Morpholino)ethanesulfonic acid
M6PR	Mannose 6-phosphate receptor (cation-dependent)
min	Minutes
NCS	New born calf serum
NIDDM	Non insulin dependent diabetes mellitus
NSF	N-ethylmaleimide sensitive factor
OD	Optical density
O/N	Over night
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDK	Phosphoinositide dependent protein kinase
PEG	Polyethylene glycol
PH	Pleckstrin homology
PI3 kinase	Phosphatidylinositol 3-kinase
PI4 kinase	Phosphatidylinositol 4-kinase
PIP_2	Phosphatidylinositol 3,4-bisphosphate
PIP_3	Phosphatidylinositol 3,4,5-trisphosphate

PKB	Protein kinase B
PM	Plasma membrane
PTB	Phosphotyrosine binding
s	Seconds
SCAMPs	Secretory carrier-associated membrane proteins
SDS	Sodium dodecyl sulphate
SH2	Src homology 2
SH3	Src homology 3
SNAP25/23	Synaptosome-associated protein 25/23
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
SP	Soluble protein
TBST-1	Tris buffered saline Tween
TEMED	N, N, N', N'-tetramethylethylenediamine
Tf-HRP	HRP-conjugated transferrin
TfR	Transferrin Receptor
TGN	Trans-Golgi network
Trp	Tryptophan
Ura	Uracil
UV	Ultra violet
VAMP	Vesicle-associated membrane protein
v/v	volume/volume ratio
w/v	weight/volume ratio

CHAPTER 1 - INTRODUCTION

1.1 Insulin action in fat and muscle

Insulin is a key hormone in regulation of fuel metabolism. It is secreted by the β cells of the pancreas in response to high blood glucose levels. One of the main actions of insulin is on muscle and adipose tissue and this will be discussed in detail here, but insulin also suppresses gluconeogenesis in the liver and increases glycolysis thereby controlling liver glucose output. By these means, insulin plays a key role in whole body glucose homeostasis.

Insulin effects on cells are dependent on the presence of an insulin receptor to which insulin binds and this leads to a cascade of events, signalling to the cell the presence of high blood glucose levels. In both fat and muscle cells this causes the uptake of glucose into those cells. Here glucose is metabolised and either converted into glycogen in muscle or fatty acids in adipose tissue. Indeed both muscle and fat are the main energy stores of the body and can be used, when blood glucose levels are low. Uptake of glucose into tissues is made possible by the presence of glucose transporters in the plasma membrane. Glucose transporters belong to a family of hexose transporters, which will be discussed in detail below. The effect of insulin on muscle and fat is mediated by the glucose transporter GLUT4. This transporter is insulin-sensitive and in the absence of insulin it is localised intracellularly. GLUT4 levels at the plasma membrane increase rapidly after insulin binding its receptor and this ensures the uptake of glucose when blood glucose levels are high. A defect in GLUT4 translocation will cause a decreased

uptake of glucose due to insulin and is thought to be the underlying cause of diseases such as diabetes mellitus.

1.2 Diabetes

Diabetes mellitus can be divided into two different types. Insulin-dependent diabetes mellitus (IDDM) and non insulin-dependent diabetes (NIDDM), now called type 1 and 2 respectively. Type 1 often manifests itself in childhood and it thought to be genetically determined leading to the destruction of the β-cells in the pancreas and therefore to insulin deficiency. Type 1 can often be treated successfully with the administration of insulin. Type 2 on the other hand is often called "late onset diabetes" and it is greatly influenced by external factors such as diet and lifestyle. Both types show a phenotype of hyperglycemia, but as mentioned earlier type 1 patients produce no insulin, while type 2 patients are often hyperinsulinaemic. Type 2 as well as obesity is characterised by insulin resistance. Studies on nondiabetic offspring of two diabetic patients showed that they have a 50% risk of becoming diabetic in later life, suggesting that type 2 diabetes is also genetically determined. This risk increased greatly if the individuals were also obese suggesting an environmental component in the development of type 2 diabetes (as reviewed in Kahn 1994).

Insulin resistance precedes the development of type 2 diabetes. Patients that are diabetic have probably been insulin resistant for many years before the disease is diagnosed, usually only by the appearance of side effects. These side effects are caused by the increased blood glucose levels over a long period of time and include in extreme cases

kidney failure, blindness and cardiovascular disease. The decreased insulin sensitivity in individuals often leads to an increase in insulin production, which again might further reduce insulin sensitivity. Often though, before the onset of diabetes, insulin secretion is severely impaired. In such patients, insulin treatment is only sometimes successful due to the fact that the patients are insulin resistant, therefore there is a great need for drugs which can alleviate this by increasing insulin sensitivity.

A lot of research has therefore gone into finding the molecular basis for insulin resistance. Any defect in the insulin signalling cascade or in GLUT4 translocation could lead to the development of insulin resistance. Studies using human tissue as well as rodent models of diabetes have revealed that there is probably not just one point in the cascade of events which is defective. It could be an accumulation of different defects, and there might be different reasons for the development of insulin resistance in different patients.

It is now known that a defective glucose transport is the main reason for insulin resistance in type 2 patients (Bonadonna *et al.* 1993; Bonadonna *et al.* 1996; Kelley *et al.* 1996). These data suggest a defect in the translocation of GLUT4 to the plasma membrane, leading to a reduced number of transporters in the plasma membrane. Indeed impaired GLUT4 translocation has been found in muscle of obese, insulin resistant and type 2 diabetic patients (Kelley *et al.* 1996; Garvey *et al.* 1998).

Muscle tissue accounts for the majority of post prandial glucose uptake at about 70-80% compared to 5-20% in adipose tissue (as reviewed in Hunter & Garvey 1998). Downregulation of GLUT4 in those tissues could explain the decreased glucose

transport in diabetic patients and indeed in adipocytes GLUT4 levels are down by about 80-90% (Garvey *et al.* 1991). On the other hand GLUT4 levels in muscle are unchanged (Garvey *et al.* 1992) and GLUT4 depletion can therefore not be the only reason for insulin resistance in type 2 diabetes. The defect in GLUT4 translocation in muscle therefore could be due to either a defect in the translocation mechanism of GLUT4 or due to mis-targeting of GLUT4 to an insulin insensitive location. Diabetes due to impaired GLUT4 recycling will be discussed in more detail in section 1.5.5.

In this study we are going to examine GLUT4 trafficking defects in an insulin resistant model system (Chapter 4). We find here that proteins involved in GLUT4 translocation are altered as well as some evidence for GLUT4 mis-targeting in insulin resistant cells.

1.3 Insulin signalling

Insulin is the only hormone known to regulate GLUT4 translocation. Therefore some understanding of the insulin-signalling cascade is necessary to study this mechanism. Below, the present state of understanding of this rapidly moving field will be reviewed.

1.3.1 The insulin receptor

The insulin receptor is expressed ubiquitously and is made of two α and two β subunits (reviewed in Virkamäki *et al.* 1999; Elmendorf & Pessin 1999). These form a heterotetramer linked via disulphide bonds. The α subunits are extracellular and contain the ligand binding domain. The β subunits span the membrane but the majority of this

polypeptide is located within the cytosol. This receptor belongs to the tyrosine kinase family of receptors, and the intracellular β subunits contain tyrosine kinase activity (reviewed in Kahn 1994; Virkamäki *et al.* 1999). Binding of insulin to the α subunits causes a conformational change in the receptor that increases the intrinsic kinase activity and leads to the autophosphorylation of the receptor β subunits (reviewed in Kahn 1994). Mutational studies of the tyrosine residues on the receptor that are phosphorylated in response to insulin have identified which residues are important for the activation of certain downstream pathways. Autophosphorylation of the receptor is known to be associated with increased tyrosine kinase activity towards endogenous substrates (reviewed in Holman & Kasuga 1997).

1.3.2 IRS

The most important downstream targets for the insulin receptor in terms of glucose transport are the "insulin receptor substrate" proteins (IRS) (Quon *et al.* 1994; Zhou *et al.* 1997). These belong to a family of at least 4 proteins of which IRS-1 and IRS-2 are thought to be the major mediators of insulin action (Previs et al. 2000; and reviewed in Elmendorf & Pessin 1999). All IRS proteins have a similar overall structure, containing a N-terminal pleckstrin homology (PH) domain and a phosphotyrosine binding domain (PTB), both of which are required for efficient tyrosine phosphorylation of IRS by the insulin receptor (Virkamäki *et al.* 1999). The PH domain recognises polar lipids in the membrane and it is known to be important in targeting IRS to the membrane and mediating association with the receptor (as reviewed in Shepherd *et al.* 1998;Virkamäki *et al.* 1999). The PTB domain recognises phosphotyrosine in the amino acid sequence NPXpY that is present in the insulin receptor. This leads to binding of IRS to the insulin

receptor and the subsequent phosphorylation of IRS on tyrosine residues (Kublaoui *et al.* 1995; Inoue *et al.* 1998). All IRS proteins contain multiple tyrosine residues many of which are located in the consensus sequence YMXM (reviewed in White & Kahn 1994). Tyrosines in this sequence are the preferred substrates of the insulin receptor and after phosphorylation the phosphotyrosine in this motif is recognised and bound by downstream targets (as reviewed in Shepherd *et al.* 1998). Association of IRS with the insulin receptor is only transient and after dissociation IRS can be recognised by proteins containing Src homology-2 (SH2) domains. SH2 domains are phosphotyrosine binding structures which recognise phosphotyrosine in the context of specific amino acid sequences (as reviewed in Virkamäki *et al.* 1999). There are many proteins that contain such SH2 domains and hence can bind to phosphorylated IRS. The most important for glucose transport is PI3-kinase (Zhou *et al.* 1997) and will be discussed in detail below. In general, SH2 domains have a higher affinity for phosphotyrosines than PTB domains. Hence, the insulin-dependent phosphorylation of tyrosine residues in IRS proteins represents a key early signal in the action of insulin.

Generation of IRS-1 and IRS-2 knockout mice has greatly helped to establish the importance and function of these proteins. Mice lacking IRS-1 are severely growth retarded, but not diabetic, compensating for insulin resistance by increased insulin secretion (Araki *et al.* 1994; Tamemoto *et al.* 1994). This data revealed that insulin signalling can be independent of IRS-1 and led to the discovery of IRS-2 (Araki *et al.* 1994). IRS-2 knockout mice on the other hand are diabetic due to insulin resistance both in muscle and liver as well as a defect in β-cell function (Withers *et al.* 1998).

When carbohydrate and lipid metabolism was studied *in vivo* in these animals, it was found that IRS-1 knockout mice were insulin resistant mainly due to decreased glycogen synthesis in muscle, suggesting that IRS-1 has its major role in this tissue. In IRS-2 knockout mice there were defects in muscle and fat metabolism as well as liver glucose metabolism suggesting a role of IRS-2 in all three tissues. These data demonstrate that the development of glucose intolerance requires defects in multiple sites including muscle, liver and adipose tissue (Previs *et al.* 2000).

The majority of both IRS-1 and IRS-2 is found in the low density fraction in 3T3-L1 adipocytes (Heller-Harrison *et al.* 1995) where they are thought to be associated with the cytoskeleton rather then membranes (Clark *et al.* 1998; Clark *et al.* 2000). It is thought that this localisation is important for the association and subsequent phosphorylation of IRS-1 and 2 by the insulin receptor, and helps to recruit PI3-kinase from the cytosol to the cytoskeleton. This in return may localise this enzyme to its membrane bound substrates. Acute insulin treatment causes the release of IRS-1 into the cytosol (Heller-Harrison *et al.* 1995; Ricort *et al.* 1996; Clark *et al.* 2000). This translocation of IRS-1 to the cytosol is probably regulated by serine/threonine phosphorylation of IRS-1 (Heller-Harrison *et al.* 1995; Inoue *et al.* 1998) and might function as an "off" switch for IRS-1.

1.3.3 PI3-kinase

PI3-kinase is a lipid kinase and a key player in mediating insulin effects on metabolism. PI3-kinases can be divided into three classes, but only class 1a is thought to mediate the common insulin responses such as glucose transport, therefore only this class will be discussed here. PI3-kinases consist of two subunits, both of which have several isoforms

(as reviewed in Shepherd *et al.* 1998). The adaptor/regulatory subunits are between 50 and 85 kDa and contain 2 SH2 domains, an SH3 domain for interaction with other proteins, as well as a domain for binding the p110 (catalytic) subunit. As discussed below, p85 is the adaptor important in GLUT4 translocation. p110 is the catalytic subunit and contains a p85 binding domain as well as the kinase domain. PI3-kinase phosphorylates the inositol ring at the D-3 position thereby generating PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$.

Insulin-stimulation causes the recruitment of p85 to IRS (Kelly & Ruderman 1993) via the binding of the SH2 domain to phosphotyrosine residues on IRS present within the YMXM motif mentioned above (Figure 1.1). This results in an increased PI3-kinase activity (Kelly *et al.* 1992) by stimulating p110, and the subsequent formation of $PI(3,4)P_2$ and $PI(3,4,5)P_3$ in both adipocytes and muscle (Kelly & Ruderman 1993; Navé *et al.*1996; van der Kaay *et al.* 1997). The increase in these phospholipids in membranes following insulin challenge causes the activation and recruitment of other proteins leading ultimately to an increase in glucose transport (see below).

Functional data has shown the importance of PI3-kinase in glucose transport. Experiments using the PI3-kinase inhibitors wortmannin and LY294002 have shown that PI3-kinase is crucial for insulin mediated glucose uptake (Okada *et al.* 1994; Cheatham *et al.* 1994; Clarke *et al.* 1994). Wortmannin inhibits the exocytosis of GLUT4 (Yang *et al* 1996) and has been shown to completely abolish the insulin-stimulated increase in PIP_2 (Heller-Harrison *et al.* 1996) and PIP_3 (van der Kaay *et al.* 1997; Heller-Harrison *et al.* 1996). Overexpression of a mutant p85, which lacks the p110-binding site caused decreased PIP_3 levels as well as impaired insulin-induced

glucose transport in CHO cells (Hara *et al.* 1994). Similarly the microinjection of either the SH2 domain of p85 fused to GST or an antibody to p110 into 3T3-L1 adipocytes inhibited insulin-stimulated GLUT4 translocation by about 75% (Haruta *et al.*1995; Hausdorff *et al* 1999). A constitutive active form of PI3-kinase has been shown to cause GLUT4 translocation in adipocytes to a similar amount as insulin (Tanti *et al* 1996).

Recently studies have provided new insight into the role of PIP_3 in glucose transport (and therefore indirecty the role of PI3-kinase). Firstly, expression of the SH2 domain containing inositol phosphatase (SHIP), a 5'phosphatase, in 3T3-L1 adipocytes decreased the intracellular levels of PIP_3 and inhibited insulin-induced GLUT4 translocation by at least 65% (Vollenweider *et al* 1999). These data indicate that PIP_3 is a key phospholipid in insulin-stimulated GLUT4 translocation.

Using synthetic PIP_3 analogues introduced into permeabilised 3T3-L1 adipocytes, Jiang and colleagues found that these compounds did not have any effect on glucose transport when administered alone, but could overcome wortmannin inhibition of glucose transport, thereby restoring 90% of the insulin stimulation (Jiang *et al* 1998). The authors conclude from these results, that PIP3 is necessary, but not sufficient for glucose transport.

Collectively, these experiments confirm the need of a functional PI3-kinase in GLUT4 translocation, but also suggest that there is a second PI3-kinase independent pathway necessary for glucose transport, (see section 1.3.6).

Interestingly other growth factors such as PDGF can also slightly increase glucose transport, but do not cause the translocation of GLUT4 from intracellular stores to the plasma membrane (Navé *et al.* 1996; Ricort *et al.* 1996). Both insulin and PDGF lead to the activation of PI3-kinase in 3T3-L1 adipocyte (Wiese *et al.* 1995), but the location of this activation is different for the two growth factors. In the basal state PI3-kinase is found at similar levels in the cytosol, plasma membrane and low density microsomes, the fraction which also contains the majority of GLUT4. PDGF decreased the cytosolic fraction of p85 and increased its amount at the plasma membrane. Insulin on the other hand causes the translocation of p85 from the cytosol to the low density microsomes (Navé *et al.* 1996; Ricort *et al.* 1996). Similarly PDGF causes an increase of PI3-kinase activity in the plasma membrane fraction, while insulin mainly causes increased PI3-kinase activity in the LDM fraction (Navé *et al.* 1996; Ricort *et al.* 1996; Clark *et al.* 1998). These differences in subcellular localisation of activated PI3-kinase might explain how PDGF and insulin can cause different downstream responses using the same central enzyme.

A peptide mimicking the phosphorylated YMXM motif of IRS-1 has been shown to activate PI3-kinase to the same extent as insulin, but was only able to stimulate glucose transport to about 20% of the level of insulin (Herbst *et al.* 1995). This again highlights the importance of the correct cellular location of activated PI3-kinase triggered by the association with IRS.

The association of PI3-kinase with the LDM fraction prompted several laboratories to examine where PI3-kinase is located in this fraction. One study has reported PI3-kinase in GLUT4 vesicles (Heller-Harrison *et al.* 1996), but it is now thought that the majority

of PI3-kinase in this fraction is not membrane associated, but attached to the cytoskeleton. The significance of this is not clear yet, but it is thought that the arrangement of signalling proteins on a scaffold may help them to interact with each other and position them at the right cellular location (Clark *et al.* 1998).

1.3.4 PKB and atypical PKC

As mentioned above PIP_3 is the major product of PI3-kinase *in vivo* in insulin-stimulated cells (van der Kaay *et al.* 1997). Many proteins can bind PIP_3 via their PH domain and this may recruit or activate these proteins. One such protein is protein kinase B (Akt) a serine/threonine kinase that can be activated by a range of growth factors. Insulin has been shown to activate PKB in a wortmannin sensitive manner (Wijkander *et al.* 1997; Shepherd *et al.* 1997). There are three isoforms of PKB and all of these are activated by PI3-kinase (as reviewed in Shepherd *et al.* 1998). Another protein containing a PH domain is 3'-polyphosphoinositide-dependent protein kinase (PDK1), which is thought to activate PKB by phosphorylation (Alessi *et al.* 1997). PKB binding to the membrane via its PH domain causes a conformational change, required for phosphorylation by PDK1. Phosphorylation on a serine and a threonine residue will cause the activation of PKB, which then enables PKB to translocate to its target site. PKB is known to be involved in many cellular responses such as differentiation, prevention of apoptosis and glucose metabolism (as reviewed in Shepherd *et al.* 1998).

Data concerning the function of PKB in glucose transport is ambiguous. A dominant negative form of PKB, which inhibits endogenous PKB, had no effect on glucose transport (Kitamura *et al.* 1998). In the same study a dominant negative form of PI3-

kinase was found to inhibit both PKB and glucose transport. A constitutively active form of PKB on the other hand has been shown to cause GLUT4 translocation in 3T3-L1 adipocytes to nearly the same extent as insulin (see for example Kohn *et al.* 1996). Microinjection of either a PKB substrate peptide or PKB antibody into 3T3-L1 adipocytes was found to inhibit insulin-stimulated GLUT4 translocation by ~ 60% (Hill *et al.* 1999). Furthermore, these authors suggest that insulin-stimulated PKB activity in 3T3-L1 adipocytes mainly involves the PKBβ isoform. This isoform is upregulated during adipocyte differentiation, and appears to be only poorly activated by PDGF in these cells, perhaps offering a further clue to the specificity of response to these different agonists (Hill *et al.* 1999). The only partial inhibition mentioned above suggests that although PKB is necessary for GLUT4 translocation, there is also a PKB-independent pathway in insulin action.

Other proteins that can bind PIP_3, although not via a PH domain, are the atypical protein kinase C isoforms (aPKC). PKCs are serine/threonine kinases and many different isoforms of this kinase have been found and classified into subfamilies (as reviewed in Mellor & Parker 1998). The conventional (cPKC) and the novel (nPKC) PKCs are regulated by diacylglycerol or phorbol ester. These isoforms of PKC are not thought to be important in insulin stimulated glucose transport (Gibbs *et al.* 1991; Merrall *et al.* 1993; Bandyopadhyay *et al.* 1999) and will not be discussed further here. The atypical PKCs are not activated by diacylglycerol or phorbol ester, but can be activated by insulin and may play a role in glucose transport. The insulin activation of both atypical PKC-λ and PKC-ζ is dependent on PI3-kinase and the formation of PIP_3. PKCs can bind PIP_3 via a mechanism not yet determined, and they are thought to be activated further by phosphorylation by PDK-1 as well as by autophosphorylation (Standaert *et al.* 1997; Le

Good *et al.* 1998; Standaert *et al.* 1999). Overexpression of PKC-ζ caused some changes in the cellular distribution of GLUT1 and GLUT4 in 3T3-L1 adipocytes (Bandyopadhyay *et al.* 1997) and in rat adipocytes a constitutivly active PKC-ζ increased insulin-stimulated GLUT4 translocation (Bandyopadhyay *et al.* 1999). A dominant-negative PKC-ζ inhibited both basal and insulin-stimulated glucose transport in 3T3-L1 adipocytes (Bandyopadhyay *et al.* 1997) and inhibited insulin stimulated GLUT4 translocation in rat adipocytes by about 60 - 70% (Standaert *et al.* 1997; Bandyopadhyay *et al.* 1999). Similarily a dominant negative PKC-λ also inhibited insulin stimulated GLUT4 translocation and this could be reversed by wild type PKC-ζ and vice versa (Bandyopadhyay *et al.* 1999). These data therefore suggest that both atypical PKCs might function in supporting insulin stimulated GLUT4 translocation in rat adipocytes. Interestingly insulin also increased the levels of both PKC-ζ and PKC-λ on GLUT4 vesicles in rat adipocytes (Standaert *et al.* 1999), but the significance of this is not yet known.

So far, little is known about the downstream targets of PKC leading to glucose transport. A recent study found that PKC-ζ could phosphorylate IRS-1 and that this might be important in a negative feedback pathway (Ravichandran *et al.* 2001).

1.3.5 Other PI3-kinase targets

There are also many other targets of PI3-kinase products. Early-endosomal antigen EEA1 is known to bind PI(3)P via its zinc-finger domain (Patki *et al.* 1998). This protein is a rab5 effector and therefore links PI3-kinase to rab5 (Simonsen. *et al.* 1998). Rab5 is

a small GTPase which is known to be important in docking and fusion of early endosomes. EEA1 will only bind rab5-GTP, the active form of rab5 and it is suggested that it is recruited to early endosomes and further stabilised by binding of EEA1 to PI(3)P (Simonsen *et al.* 1998). Interestingly it was found that rab5-GTP also binds directly to the catalytic subunit of one isoform of PI3-kinase and that this further helps to recruit EEA1 (Christoforidis *et al.* 1999). EEA1 is known to be important in specifying vesicle targeting to early endosomes.

So far no equivalent mechanism has been found for the insulin-stimulated products of PI3-kinase on GLUT4 vesicles. Rab4 is known to be important in insulin-mediated GLUT4 translocation (Vollenweider *et al.* 1997) and the activation of PI3-kinase by insulin leads to an increase in the GTP loading of rab4 (Shibata *et al.* 1997). Rab4 has been implicated as a potential insulin-regulated switch involved in the release of GLUT4 containing vesicles from their intracellular location (Cormont *et al.* 1996; Mora *et al.* 1997). It remains to be determined how rab4 and its effectors contribute to the translocation mechanism of GLUT4.

1.3.6 An alternative pathway of insulin signalling - Cbl/CAP complex

Many of the studies described above using introduction of different signalling proteins only found a partial inhibition of insulin action. This prompted a search for an alternative pathway of insulin signalling involved in glucose transport.

Cbl is the cellular homologue of the v-Cbl onco-protein. It is approximately 120kDalton in size, predominantly cytosolic and contains many proline-rich motifs with which it can

bind SH3 domains on other proteins. Insulin has been shown to tyrosine phosphorylate Cbl in 3T3-L1 adipocytes (Ribon & Saltiel 1997) and this can lead to the binding of Cbl with several other proteins. In B cells, Cbl was found to bind p85 and it is therefore thought that it might be important in PI3-kinase recruitment and activation in these cells (Kim *et al.* 1995). In 3T3-L1 adipocytes on the other hand, tyrosine phosphorylated Cbl was unable to bind PI3-kinase (Ribon & Saltiel 1997). It is now thought that Cbl can bind the insulin receptor via the Cbl associated protein (CAP). Interaction of CAP with Cbl is mediated by binding of the SH3 domains in CAP to the proline rich motifs in Cbl and is independent of insulin action. CAP also binds to the insulin receptor and insulin stimulation causes a time-dependent dissociation of the CAP-insulin receptor complex (Ribon *et al.* 1998). After insulin-mediated dissociation of the CAP-Cbl complex from the insulin receptor, the complex then moves to caveolin-enriched triton-insoluble membrane fractions (Baumann *et al.* 2000). More interesting is the observation that the introduction of CAP lacking the SH3 domains (CAPΔSH3) into 3T3-L1 adipocytes had no effect on PI3-kinase activity, but was found to inhibit insulin-mediated GLUT4 translocation by ~ 50%-60% (Baumann *et al.* 2000). A recent study has found that the insulin-mediated translocation of CAP/Cbl to lipid raft domains in the plasma membrane activates a small GTP-binding protein TC10, but so far it is not known how this protein affects glucose transport (Chiang *et al.* 2001). These results suggest that insulin can generate two independent signals leading to glucose transport, one dependent on and the other independent of PI3-kinase.

1.3.7 Insulin signalling in diabetes

Clearly defects in insulin signalling could lead to insulin resistance and diabetes (as reviewed in Virkamäki *et al.* 1999; Saltiel 2001). Although rodent models are important in studying insulin resistance and have been used extensively, very few are a real model of type 2 diabetes with the same phenotype as that found in human diabetes. Therefore recent studies have concentrated on finding signalling defects in diabetic patients. Signalling defects that cause insulin resistance are rare, but some will be mentioned here. On the other hand it is well established that there are several acquired defects in insulin resistance. In type 2 patients a downregulation of insulin receptor as well as decreased tyrosine kinase activity have been found, but this can be restored by weight loss (Freidenberg *et al.* 1988). Decreased IRS-1 expression has also been found in adipocytes of insulin resistant patients, yet interestingly IRS-2 was found to substitute for IRS-1 and the main insulin-stimulated PI3-kinase activity was now associated with IRS-2 (Rondinone *et al.* 1997). On the other hand in skeletal muscle no changes in IRS-1 expression was observed, but decreases in its tyrosine phosphorylation in response to insulin have been reported, together with a defect in insulin stimulated PI3-kinase activity in type 2 diabetic patients (Bjornholm *et al.* 1997). Another study reported a 50% decrease in IRS-1 associated PI3-kinase activity and a 39% decrease in IRS-2 associated PI3-kinase activity in muscle of type 2 diabetic patients (Kim *et al.* 1999). PKB activity and levels have been shown to be unchanged in muscle of diabetic patients and this may suggest that PKB is not a major factor in the development of insulin resistance (Kim *et al.* 1999).

Genetic defects in insulin signalling proteins, such as the insulin receptor are very uncommon and only found in some syndromes such as leprechaunism and type A insulin resistance (reviewed in Hunter & Garvey 1998). There are several polymorphisms occurring in IRS-1 which are increased in type 2 diabetic patients. These do not lead to a large defect on IRS-1 function, but decreased PI3-kinase activity has been reported in some (Almind *et al.* 1996; Imai *et al.* 1997). To date the only mutation found in PI3-kinase is present at the same frequency in diabetics and normal individuals and it is therefore unlikely to be important in the development of insulin resistance and type 2 diabetes (as reviewed in Shepherd et al. 1998; Virkamäki *et al.* 1999).

1.4 Glucose transport

Glucose is the major energy source for most mammalian cells and, in order to be used in the cytosol, has to be transported across the plasma membrane.

There are two major types of glucose transporters. The sodium-dependent transporters use a Na^+ gradient to drive accumulation of glucose (as reviewed in Bell *et al.* 1993), and will not be discussed further here, and the facilitative glucose transporters that move glucose down its chemical gradient and do not use energy for this transport.

1.4.1 The family of glucose transporters

There are several isoforms of the facilitative glucose transporters. They are products of distinct genes, exhibit tissue-specific expression, and are independently controlled. The

facilitated glucose transporters are integral membrane proteins and their structure will be discussed in some detail below.

Seven different functional facilitative sugar transporters have been characterised in mammals.

GLUT1 was the first to be isolated from red blood cells and is ubiquitously expressed. It is the major transporter at the blood-tissue barriers and provides cells with glucose even if extracellular glucose levels are low. GLUT1 is sometimes called the "house-keeping" glucose transporter as it provides tissues with a constant low level of glucose (as reviewed in Gould 1997).

GLUT2 is a bi-functional glucose/fructose transporter and is mainly expressed in the liver, the beta cells of the pancreas and the small intestine. In the liver, it is important for both import and export of glucose, depending on the blood glucose levels and thereby ensuring the important role of the liver in whole body glucose homeostasis (as reviewed in Gould & Holman 1993; Gould 1997).

GLUT3, the neuronal glucose transporter is crucial for supplying the brain with glucose as brain has a high glucose demand and can not use any other energy source. As such it has the lowest Km of all the transporters studied functionally to date (as reviewed in Bell *et al.* 1993).

GLUT4, the "insulin-sensitive glucose transporter" is the most intensively studied member of the family (as reviewed in Rea & James 1997). It is expressed in muscle,

heart and fat tissue and undergoes insulin-mediated translocation from an intracellular store to the plasma membrane thereby ensuring the insulin responsiveness of those tissues. This leads to the uptake of glucose into glucose storage tissues and subsequently glycogen is synthesised in muscle and fatty acids and triglycerides are made in fat cells. This transporter is discussed further below.

GLUT5 is a fructose transporter and although expressed in many tissues it is highly expressed in the small intestine where it is thought to be important in mediating fructose transport from the intestine lumen (as reviewed in Gould 1997).

GLUT6 does not encode a functional glucose transporter, it being a pseudogene with several stop codons (as reviewed in Gould & Holman 1993). GLUT7 has been withdrawn from the database.

More recently several new members have been cloned. The newly identified GLUT8 encodes a functional glucose transporter which has a wide tissue distribution with highest levels in testis. Interestingly its expression in testis was suppressed by estrogen treatment and it is thought that this transporter might have a role in the provision of glucose in male germ cells (Doege *et al.* 2000). So far little is known about GLUT9, but its expression was found to be highest in kidney and liver (Phay *et al.* 2000).

1.4.2 Structure

Analysis of the hydropathy plots of the predicted amino acid sequences of the different isoforms has shown that these proteins adopt similar structures (Figure 1.2). These

studies predict an integral membrane protein with twelve amphipathic helices spanning the membrane with both the N- and C-terminus on the cytoplasmic side. There are large loops between helices 1 and 2 (extracellular) and 6 and 7 (intracellular) (as reviewed in Gould & Holman 1993). The major features of this model have been experimentally confirmed (as reviewed in Gould 1997) and it is thought that five of the 12 helices form an aqueous channel through which glucose can traverse the membrane (as reviewed in Barrett *et al.* 1999). Conformational studies using chimeras of the different glucose transporters isoforms have allocated specific functions to certain sequences, structures or even amino acids within these transporters. The substrate binding site is known to be made up of different regions of the transporter and binding of substrate to the exofacial site causes a conformational change re-orientating the sugar binding site to the endofacial conformation and subsequent release of the substrate into the cytoplasm (as reviewed in Gould 1997; Barrett *et al.* 1999).

1.5 GLUT4

1.5.1 The translocation hypothesis

In contrast to the other glucose transporter isoforms, which are located mainly at the plasma membrane, 95 % of GLUT4, the "insulin-sensitive glucose transporter", is found in intracellular vesicles and tubules (Slot *et al.* 1991a; Slot *et al.* 1991b). Insulin causes the translocation of these vesicles to the cell surface resulting in up to 20-fold increased levels of GLUT4 at the plasma membrane and therefore increased glucose transport (as reviewed in Rea & James 1997).

The intracellular location of GLUT4 and the mechanism of its translocation will be discussed in detail below.

1.5.2 Localisation by EM

In the last years much research has gone into examining the nature of the GLUT4 containing compartment. Electron microscopy studies, using brown adipose tissue or rat cardiac muscle, have shown GLUT4 to be localised to about 40% in tubulo-vesicular structures near the Golgi, some of this probably being TGN. Another 50% was found in tubulo-vesicular structures scattered throughout the cytoplasm (Slot *et al.* 1991a; Slot *et al.* 1991b). GLUT4 localisation in tubulo-vesicular structures both near the TGN as well as throughout the cytoplasm has also been confirmed in skeletal muscle (Ploug *et al.* 1998). The presence of GLUT4 near the TGN led to experiments using rat atrial cardiomyocytes, a secretory cell type, in order to determine whether GLUT4 is actually localised in this compartment. In these cells a large proportion of GLUT4 (50 - 60%) was found in the secretory granules which indicates that GLUT4 traffics through the TGN during recycling (Slot *et al.* 1997). However, GLUT4 vesicle purification studies argue against the TGN being the major intracellular storage compartment in adipocytes (Martin *et al* 1994).

In brown adipose tissue GLUT4 levels at the plasma membrane were observed to be around 1% in the absence of insulin, confirming the idea that GLUT4 is excluded from the cell surface in the absence of stimulation. Insulin then caused a redistribution of GLUT4 from these intracellular structures to the plasma membrane by about 40% (Slot

et al. 1991a; Slot *et al.* 1991b). Insulin stimulation also resulted in increased labelling of GLUT4 in clathrin lattices and early endosomes, suggesting that GLUT4 is endocytosed via clathrin coated pits, a well described mechanism for other membrane proteins (as reviewed in Robinson *et al.* 1996).

Recently a new method for the isolation of cytoplasmic sheets from rat adipocytes has allowed a 3-D resolution of the intracellular GLUT4 compartments using electron microscopy (Ramm *et al.* 2000). In this study the majority of GLUT4 was found in vesicles (60%) while a smaller part of GLUT4 was in tubules (25%). Interestingly insulin caused a recruitment of GLUT4 mainly from the small vesicles and translocation of GLUT4 is though to occur via fusion of these vesicles with the plasma membrane rather then sorting of GLUT4 out of the vesicles (Ramm *et al.* 2000).

The presence of GLUT4 in different intracellular compartments will be discussed in more detail below.

1.5.3 Multiple compartments

The main effort in the last years therefore has been to identify what compartment these tubulo-vesicular structures are. One obvious suggestion was that GLUT4 is part of the constitutive recycling pathway. It is known that GLUT4 is endocytosed via clathrin coated pits (Slot *et al.* 1991a; Robinson *et al.* 1992) and recycles constantly from the plasma membrane to intracellular sites both in the presence and absence of insulin (Robinson *et al.* 1992; Yang & Holman 1993). GLUT4 partially colocalises with several other proteins such as GLUT1 (Calderhead *et al.* 1990; Robinson & James 1992), the

mannose-6-phosphate receptor (M6PR) (Kandror & Pilch 1996) and the transferrin receptor (TfR) (Tanner & Lienhard 1989) in endosomes. These proteins also undergo insulin responsive translocation to the cell surface in 3T3-L1 adipocytes (Tanner & Lienhard 1987; Tanner & Lienhard 1989; Calderhead *et al.* 1990; Kandror & Pilch 1996; Kandror & Pilch 1998; Subtil *et al.* 2000), however this increase is only about 2-fold compared to the ~ 20-fold increase of GLUT4 (Tanner & Lienhard 1987; Calderhead *et al.* 1990; Piper *et al.* 1991). This suggests that although GLUT4 is partly present in the same compartment as those proteins, a significant fraction is localised to a compartment that is distinct from endosomes and which is highly responsive to insulin (Yang & Holman 1993).

The idea that GLUT4 populates at least two different intracellular compartments (endosomes and GSVs) has been suggested by data using many different methods. From a kinetic point of view, the behaviour of GLUT4 recycling kinetics can only be explained by the presence of at least two intracellular GLUT4 pools (Holman *et al.* 1994). In this study the membrane impermeant bismannose photolabel ATB-BMPA was used to tag GLUT4 at the plasma membrane after insulin treatment, and the subsequent re-distribution of the tagged GLUT4 was examined. The behaviour of tracer-tagged GLUT4 was then fitted into different models for GLUT4 recycling (Figure 1.3). The 3-pool model with two intracellular pools, one early endosome pool (T_{ee}) and one tubulo-vesicular pool (T_{tv}), can account for the above mentioned differences in recycling between GLUT4 and other proteins. It also allows for a rapid translocation of GLUT4 to the plasma membrane and fits into the observation that the main effect of insulin is to increase the exocytosis rate of GLUT4 rather then decrease the endocytosis rate (Slot *et al.* 1991a; Yang & Holman 1993). This model involves only one plasma membrane pool

of glucose transporter and therefore does not account for the presence of GLUT4 in clathrin coats (Slot *et al.* 1991a; Robinson *et al.* 1992) or more importantly for the time-differences observed between appearance of GLUT4 at the plasma membrane and increase in glucose transport (Holman *et al.* 1994). Therefore two different 4-pool models using occluded pools were proposed, where T_{po} (Figure 1.3C) represents vesicles that have docked with the plasma membrane, but not yet fused and T_{pc} (Figure 1.3D) represents GLUT4 in clathrin coated pits ready for endocytosis. It is important to note here that the ATB-BMPA can access GLUT4 in the occluded pool. The authors of this paper propose that the only possible way to explain all the data about GLUT4 trafficking is through a 5-pool or multiple-pool model, which contains the two internal pools as well as the occluded pools (Holman *et al.* 1994).

The recently developed method to visualise the GLUT4 compartments in a 3-dimensional way has greatly helped to distinguish between the intracellular GLUT4 pools (Ramm *et al.* 2000). As mentioned above, the majority of GLUT4 was targeted to small vesicles and this vesicular compartment was shown to be distinct from early and late endosomes as well as TGN. Co-localisation studies suggested on one hand that a subset of these vesicles fuse with the plasma membrane in response to insulin, but also that GLUT4 is found in mannose-6-phosphate receptor (M6PR) positive vesicles and that insulin induced a sorting of GLUT4 out of those vesicles.

The presence of two intracellular GLUT4 pools, one being endosomes and the other highly insulin responsive, has also been confirmed biochemically and some of these experiments will be described here.

Studies in rat adipocytes have suggested that GLUT4 containing vesicles are structurally as well as functionally different from other intracellular endosomes (Kandror *et al.* 1995). Many other studies have tried to separate the insulin-sensitive GLUT4 storage vesicles (GSVs) from the endosomal GLUT4 pool. Indeed sucrose density gradient studies, which exploit the different density properties of membranes, found two pools containing GLUT4 in rat skeletal muscle. Only one of the pools showed a 30% decrease in GLUT4 levels due to insulin, while the other pool contained TfR (Aledo *et al.* 1997). The use of hypotonic lysis instead of homogenisation as a method to fractionate cells has also allowed the separation of strongly insulin-sensitive vesicles (presumably GSVs) from the less insulin-sensitive endosomes (Lee *et al.* 1999). The best method so far to separate GLUT4 in internal membranes into endosomes and GSVs is the recent use of iodixanol as a gradient material (Hashiramoto & James 2000) and this will be discussed in detail in Chapter 3.

Another method, which has given similar results, is compartment ablation. Here cells are loaded with transferrin bound to horseradish peroxidase (Tf-HRP). The Tf-HRP is taken into the cell via the transferrin receptor. Incubation of intact cells with diamino-benzidine (DAB) and hydrogen peroxide allows HRP to transfer electrons from DAB to peroxide. This produces a highly reactive cross-linker within the lumen of transferrin receptor positive vesicles/tubules, and thus any protein in proximity to the transferrin receptor is ablated (Livingstone *et al.* 1996). Both GLUT1 and the transferrin receptor were ablated to about 70% after 2h incubation with Tf-HRP while GLUT4 was found to be ablated by only 40%. This would suggest that the larger proportion of GLUT4 (60%) is localised to a TfR-negative compartment, which was also devoid of the endosomal markers rab5 and M6PR (Livingstone *et al.* 1996). This data supports the kinetic and

gradient studies mentioned earlier, and confirms the idea that GLUT4 is localised in two intracellular compartments, one being the endosomes and another being the GSVs.

1.5.4 The GSV model

The model is therefore that GLUT4 in the basal state is present in an insulin responsive compartment now termed GLUT4 storage vesicles (GSVs) (Figure 1.4). Insulin then causes a rapid translocation of GLUT4 to the plasma membrane, this occurs both via fusion of GLUT4 containing vesicles with the plasma membrane as well as sorting of GLUT4 from an endosomal compartment. From the plasma membrane GLUT4 is then endocytosed via clathrin coated pits, enters the endosomal recycling pathway, resulting in co-localisation with TfR, M6PR and GLUT1. From the endosomes a large proportion of GLUT4 is then removed into the GSVs and is stored there until a further insulin challenge.

1.5.5 GLUT4 defects in diabetes

As mentioned in section 1.2 a defective glucose transport is the main reason for insulin resistance in type 2 patients (Bonadonna *et al.* 1993; Bonadonna *et al.* 1996; Kelley *et al.* 1996). Decreased GLUT4 levels at the plasma membrane after insulin treatment could be due to defects in any stages of GLUT4 recycling and this can therefore lead to the development of type 2 diabetes.

Garvey and co-workers have extensively studied GLUT4 levels and trafficking in both adipocytes and muscle cells from type 2 diabetic patients. Although in adipocytes

GLUT4 downregulation can explain most of the decrease in insulin-stimulated glucose uptake (Garvey *et al.* 1991), there is a discrepancy between the amount of transporters found at the plasma membrane and glucose transport (Garvey *et al* 1988). Similar results were found in patients with gestational diabetes (abnormal glucose tolerance during pregnancy). In these women glucose transport rates both in basal and insulin stimulated cells were much lower than expected from the levels of GLUT4 found at the plasma membrane (Garvey *et al* 1993). This data could be explained in at least two different ways; one possibility is that in these patients there is a defect in GLUT4 activity and that GLUT4, although present at the plasma membrane, is not functional. Another explanation is that GLUT4 is not inserted correctly into the plasma membrane and therefore not transporting glucose. The authors favour this explanation and suggest that GLUT4 is mis-targeted to a compartment which in subcellular fractionations is isolated together with the plasma membrane (Garvey *et al* 1993). This could, for example, be if GLUT4 was present in an occluded pool at the plasma membrane, where GLUT4 vesicles are docked with the membrane but not yet fused. In this case GLUT4 would be isolated in the plasma membrane fraction, but not transport glucose.

A study from the same labroatory in muscle from insulin-resistant patients also suggested that GLUT4 was mis-targeted and that this could be the reason why insulin was unable to recruit GLUT4 to the plasma membrane (Garvey *et al.* 1998). Interestingly in these insulin-resistant patients GLUT4 was mis-targeted to a fraction that also contained plasma membrane and this is therefore similar to the study in adipocytes described above.

Further studies to identify the location of GLUT4 in insulin resistant cells are required.

1.5.6 GLUT4 knock out mice

The generation of a GLUT4 knock out mouse has lead to the surprising discovery that GLUT4 is not actually necessary for maintaining nearly normal blood glucose levels (Katz *et al.* 1995). These mice are growth-retarded and have decreased longevity, but are not diabetic. Examining the other glucose transporters showed that GLUT1, the only other glucose transporter present in fat and muscle tissue, was not upregulated. GLUT2 on the other hand was upregulated in liver and this is thought to indicate an increase uptake of glucose into this organ (reviewed in Mueckler & Holman 1995). The reduction of adipose tissue in these mice and the likely decrease of glycolysis in muscle, makes it unlikely that there is another hitherto unidentified glucose transporter by which these tissue transport glucose, and suggests that there is a decrease in glucose uptake into peripheral tissues. It is thought that these mice might maintain normal blood through an increased uptake of glucose into liver and thereby somewhat compensate for the lack of GLUT4.

Interestingly, heterozygous knockout mice for GLUT4 in contrast to the homozygous knockout described above, develop a diabetic phenotype (Stenbit *et al.* 1997). The reason for this is thought to be that these mice produce normal levels of GLUT4 until they are 8 weeks old, but then GLUT4 content declines. As a consequence of this heterozygous mice do not develop the compensating mechanism seen in GLUT4 null mice and therefore can not cope with the decreased glucose uptake into peripheral tissue.

A recent study examining mice deficient of GLUT4 in their adipose tissue showed that these mice developed insulin resistance (Abel *et al.* 2001). Isolated adipocytes from

those mice showed reduced glucose uptake both in the presence and absence of insulin, while muscle glucose uptake was normal. Interestingly *in vivo* glucose uptake was not only reduced in adipose tissue, but also muscle. This suggests that reduced insulin-stimulated glucose transport in adipocytes secondarily induces insulin resistance in other insulin target tissues and this might be caused by the chronic hyperinsulinaemia observed in these mice (Abel *et al.* 2001).

1.6 IRAP

IRAP or vp165 is an aminopeptidase, which was first cloned from rat adipocytes (Keller *et al.* 1995), has been found to colocalise with GLUT4 in rat adipocytes (Malide *et al.* 1997), 3T3 L-1 adipocytes (Ross *et al.* 1996) as well as rat skeletal muscle (Aledo *et al.* 1997). Immunoadsorption of GLUT4 vesicles and subsequent analysis of the vesicles with IRAP antibody as well as immunoadsorption of IRAP vesicles and subsequent analysis of the vesicles with GLUT4 antibody have shown that these two proteins colocalise perfectly in the basal state in 3T3-L1 adipocytes (Ross *et al.* 1996). Subcellular fractionations revealed that IRAP translocates to the plasma membrane to a similar extent then GLUT4 both in 3T3-L1 adipocytes (Ross *et al.* 1996) as well as rat adipocytes (Malide *et al.* 1997). Subsequently biotinylation experiments showed the increase at the plasma membrane due to insulin to be about 8-fold (Ross *et al.* 1996). Confocal microscopy of rat adipocytes using double labelling with GLUT4 and IRAP suggested an about 85% overlap between the two proteins (Malide *et al.* 1997). Similarly, using the more accurate electron microscopy method on GLUT4 or IRAP vesicles showed a 70 - 90% overlap depending on the cell type (Martin *et al.* 1997).

Ablation experiments showed that IRAP was ablated to a similar degree as GLUT4, suggesting that it is localised in two or more intracellular compartments very much like GLUT4 (Martin *et al.* 1997). Similar to GLUT4 IRAP recycles constantly between the plasma membrane and intracellular membranes and is endocytosed via clathrin coated pits (Garza & Birnbaum 2000). Several studies support the idea that GLUT4 and IRAP recycle through the endocytic pathway in a very similar manner (Malide *et al.* 1997; Ross *et al.* 1997; Garza & Birnbaum 2000).

So far very little is known about the concrete function of IRAP and it is not quite clear yet whether IRAP has any role in GLUT4 trafficking. One study has suggested a role of IRAP in constitutive, but not insulin stimulated GLUT4 trafficking (Filippis *et al.* 1998). On the other hand, injection of IRAP (especially a 28 amino acid stretch of the cytoplasmic tail of the protein) into 3T3-L1 adipocytes causes GLUT4 translocation in the absence of insulin. This translocation to the plasma membrane could not be inhibited by wortmannin the PI3-kinase inhibitor. These results therefore suggest that the action of IRAP on GLUT4 is downstream of PI3-kinase activity. (Waters *et al.* 1997) A hypothesis for these results is that retention proteins interact with the C-terminus of GLUT4 or/and the N-terminus of IRAP. Injecting IRAP peptides into cells causes competition of these peptides with intracellular IRAP for the retention proteins and thereby translocation of the GLUT4 vesicles to the plasma membrane (Waters *et al.* 1997). This data was supported by the generation of a chimeric reporter molecule vpTR that contains the N-terminal cytoplasmic domain of IRAP and the transmembrane and extracellular domains of the transferrin receptor and behaves very much like IRAP. A mutation of the di-leucine motif in the cytoplasmic tail of vpTR showed that this motif

interacts with the machinery responsible for insulin-regulated intracellular retention (Johnson *et al.* 1998; Subtil *et al.* 2000).

1.7 Other molecules in GLUT4 vesicle

1.7.1 SCAMPs

Secretory carrier membrane proteins (SCAMPs) are between 35 and 40kDa and are found in all secretory carrier membranes. They colocalise with GLUT4 in the LDM fraction both in the absence and presence of insulin, and indeed a 37kDa SCAMP is found on GLUT4 vesicles (Laurie *et al.* 1993). SCAMPs only show a slight translocation to the plasma membrane after insulin treatment and the authors propose that SCAMPs are segregated from GLUT4 in the presence of insulin possibly due to a more rapid internalisation (Laurie *et al.* 1993). A role of these proteins in GLUT4 trafficking has not been shown.

1.7.2 VAMP/Cellubrevin

Both vesicle-associated membrane protein 2 (VAMP2) and cellubrevin/VAMP3 have been found on GLUT4 vesicles (Cain *et al.* 1992; Volchuk et al. 1995; Tamori *et al.* 1996; Martin *et al.* 1996; Malide *et al.* 1997). These proteins are important for the SNARE-type mechanism of translocation of GLUT4 to the plasma membrane and will be discussed in detail below. Both VAMP2 and cellubrevin show insulin-stimulated translocation to the plasma membrane (Cain *et al.* 1992; Volchuk et al. 1995; Tamori *et*

al. 1996; Martin *et al.* 1996). It is now thought that VAMP2 is important in the translocation of the GSVs to the plasma membrane, while cellubrevin plays a role in the endosomal translocation of GLUT4 and other proteins (Volchuk et al. 1995; Cheatham *et al.* 1996; Martin *et al.* 1996; Martin *et al.* 1998).

1.7.3 PI4-kinase

PI4-kinase is a lipid kinase which phosphorylates the inositol ring at the D-4 position thereby generating PI(4)P. GLUT4 vesicles isolated form rat adipocytes have been found to contain PI 4 kinase (Del Vecchio & Pilch 1991) and indeed PI 4 kinase activity has been found on GLUT4 vesicles from rat skeletal muscle (Kristiansen et al. 1998). The kinase activity on GLUT4 vesicles from both adipocytes and muscle on the other hand is not regulated by insulin (Del Vecchio & Pilch 1991; Kristiansen et al. 1998) and this enzyme is probably not involved in GLUT4 trafficking.

1.7.4 Akt/PKB

As discussed above protein kinase Bβ (PKBβ/Akt-2) has been implicated in insulin action (Hill *et al* 1999). This isoform of PKB is located in the cytosol as well as in all membrane fractions in a subcellular fractionation of adipocytes and this is in contrast to PKBα (Akt-1) which is exclusively cytosolic (Calera *et al* 1998). Insulin has no effect on the cellular distribution of PKBα, but causes a very slight increase of PKBβ in the LDM fraction, probably deriving from the cytosol. Interestingly PKBβ was found on GLUT4 vesicles and the ratio PKBβ/GLUT4 increased after insulin treatment consistent with an increase of PKBβ and a decrease of GLUT4 in these vesicles (Calera *et al* 1998).

1.7.5 Sortilin

Sortilin is a 110 kDa protein that was found originally by its presence on GLUT4 vesicles of adipocytes (Morris *et al.* 1998). Similar to GLUT4, sortilin is absent in 3T3-L1 fibroblasts and its levels are maximal around day 8 after differentiation. Insulin causes a very small redistribution of sortilin from the LDMs to the plasma membrane in 3T3-L1 adipocytes and this is surprising due to its presence on GLUT4 vesicles. The authors of this study suggest that most of the sortilin may be in GLUT4 vesicles arising from endosomes and Golgi rather then from the GSVs (Morris *et al.* 1998). So far a role of sortilin in GLUT4 trafficking has not been found.

1.7.6 Pantophysin

The synaptophysin homologue pantophysin has been cloned from both 3T3-L1 fibroblasts (Haass *et al.* 1996) and adipocytes (Brooks *et al.* 2000). Pantophysin is an integral membrane protein of small cytoplasmic transport vesicles and it was found to be ubiquitously expressed (Haass *et al.* 1996). In 3T3-L1 adipocytes pantophysin has been shown to be localised to GLUT4 vesicles and interact with the v-SNARE VAMP2 (Brooks *et al.* 2000). Insulin caused a small increase of this protein at the plasma membrane, but incubation of permeablised 3T3-L1 adipocytes with a small C-terminal peptide of pantophysin had no effect on GLUT4 trafficking. Interestingly although insulin had no effect on the phosphorylation state of pantophysin, it stimulated the association of a 77 kDa protein with pantophysin (Brooks *et al.* 2000). These data

suggest that insulin regulates pantophysin, but the function of this protein in vesicle trafficking is still unclear.

1.8 The SNARE hypothesis

The SNARE hypothesis originates from the research on synapses, where neurotransmitters in the presynapse are stored in vesicles ready to be released into the synaptic cleft. Calcium influx into the presynapse due to an action potential travelling down the axon causes rapid release of this neurotransmitter (as reviewed in Südhof 1995). The study of these synaptic vesicles and how they fuse with their target membrane has provided much information about regulated secretion in cells in general and how this is controlled.

The SNARE hypothesis provides a conceptual framework to explain the docking and fusion of synaptic vesicles with the plasma membrane. This model states that the vesicle that is supposed to fuse with the membrane contains a protein called vesicle-SNARE (v-SNARE) and that the target membrane contains a target-SNARE (t-SNARE). Complementary v-SNARE and t-SNARE pairs are able to engage and this could initiate the fusion event (Söllner et al. 1993a). Several other proteins are also thought to be involved in this process which will be discussed below.

One of these proteins is NSF (*N*-ethylmaleimide-sensitive factor), a tetramer made of 76kDalton subunits (Block *et al.* 1988). NSF is an ATPase that can bind several other proteins in the ATP bound state (Wilson *et al.* 1992) and NSF activity is necessary for

membrane fusion (Whiteheart *et al.* 1994). In the initial experiments which led to the SNARE hypothesis Rothman and colleagues assembled the so-called 20S particle using NSF in the ATP bound state as bait. Five proteins were found to be part of the 20S complex: NSF, α-SNAP, synaptobrevin/VAMP (vesicle-associated membrane protein 2), syntaxin and SNAP25 (Söllner *et al.* 1993a and b). VAMP, syntaxin and SNAP25 are called SNAREs (for soluble NSF attachment protein receptor) and interestingly these proteins can form a complex even in the absence of α-SNAP and NSF (Söllner *et al.* 1993b). When ATP was added to this complex it was hydrolysed by NSF and the 20S particle was disassembled.

Many different isoforms of the SNAREs have subsequently been identified which has led to the suggestion that the formation of the SNARE complex is an obligatory step in all membrane fusion (Bock *et al.* 1996; Advani *et al.* 1998; Prekeris *et al.* 1998; Tang *et al.* 1998). VAMPs are located on the cytoplasmic surface of vesicles (hence the name v-SNAREs) and are anchored in the membrane via their hydrophobic C-terminus which contains a membrane spanning domain (Trimble *et al.* 1988). Syntaxin and SNAP25 located in the target or plasma membrane (Söllner *et al.* 1993b) are hence called t-SNAREs.

The original hypothesis proposed that pairing of the v-SNARE with the t-SNAREs results in vesicle docking at the plasma membrane, and in order for fusion to occur this complex has to be broken up. Due to the fact that these three SNARE proteins can form a stable entity, an energy input is required for this to happen and this was thought to be the reason why ATP hydrolysis by NSF, with the help of α-SNAP, is needed. Subsequent studies have suggested that this is an over simplified view and only correct

in parts. A more up to date model for SNARE complex function will be discussed in more detail below.

1.8.1 Evidence for the function of SNARE proteins and their specificity

Evidence in favour of the SNARE hypothesis includes experiments using the eight different botulinum and tetanus toxins. These are some of the most dangerous toxins because they inhibit synaptic vesicle exocytosis and therefore block nerve function. These toxins are proteases, five of which can cleave VAMP, two cleave SNAP-25 and one cleaves syntaxin (as reviewed in Südhof 1995). Interestingly these proteins can only be cleaved before the assembly of the SNARE complex, but not in the complex itself (Hayashi *et al.* 1994), which suggests that the cleaving sites are not accessible to the toxins once the complex has formed. Cleavage of VAMP using these toxins resulted in inhibition of neurotransmitter release in the squid giant synapse, but increased the amount of docked synaptic vesicles suggesting that VAMP was not important in the docking of the vesicles at the active zone, but necessary for a post-docking event (Hunt *et al.* 1994).

There has been considerable debate regarding the minimal machinery needed for membrane fusion. One report showed that fusion can occur when only SNARE proteins are present in the membrane without NSF and α-SNAP, although this fusion was shown to be very slow (Weber *et al.* 1998). This has led to the concept of the so-called SNAREpin model of fusion, where the SNARE proteins represent the minimal machinery required for membrane fusion. Another study showed that NSF, with the help of α-SNAP as regulator, was sufficient for rapid membrane fusion and that there was no

need for SNARE proteins (Otter-Nilsson *et al.* 1999). This latter study may be an artefact of *in vitro* experiments, or may arise as a consequence of the unusual lipid mixture of the model membranes employed. Further studies will be required to resolve these discrepancies. Nonetheless, the SNAREpin model is now becoming widely accepted.

The discovery that a SNARE type mechanism is present in all kinds of vesicle exocytosis and that there are many isoforms of the SNARE proteins prompted an analysis of the specificity of this process. In the original SNARE hypothesis it was suggested that the amount of VAMP and syntaxin isoforms present provided enough possibility for the formation of specific pairs and that this ensured the specificity of this mechanism (Söllner *et al.* 1993b). In the last years this idea has been challenged. In an *in vitro* study, SNAP-25 and two homologues, three syntaxin homologues and five VAMP isoforms were tested for the formation of a SNARE complex. Although the results demonstrated some variability, using thermostability as a measurement for complex formation, all VAMPs tested could bind a variety of t-SNARE combinations, generating stable structures (Yang *et al.* 1999). The authors of this study concluded that SNARE pairing is promiscuous and that the specificity is likely derived from other proteins that are able to regulate SNARE complex formation. Indeed, there are many proteins involved in the regulation of membrane fusion (as reviewed in Lin & Scheller 2000) some of which will be discussed below.

A recent study on the other hand showed that SNARE pairing *in vivo* is much more specific. In this study a vesicle-to-plasma membrane fusion assay was used, measuring the release of [^{3}H]norepinephrine. It is known that the SNAREs involved in this fusion

event are syntaxin1, SNAP-25 and VAMP2. Microinjection of the coiled-coil domain of both syntaxin 1 and 4, but not of any of the other syntaxin homologues could inhibit fusion of norepinephrine-containing PC12 cell granules with the plasma membrane (Scales *et al.* 2000). Similarily the coiled-coil domain of VAMP2 and to a lesser degree VAMP4 could inhibit fusion, but none of the other VAMP homologues. A modification of this assay was used to look for the ability of different SNAP-25 homologues to rescue neurotoxin cleaved SNAP-25. It was found that not only SNAP-25, but also SNAP-23 could rescue this. SNAP-29 on the other hand was much less effective (Scales *et al.* 2000). One important observation in this study, which might explain the different *in vitro* results mentioned above, is that measuring thermostability is not always a good indicator of specific SNARE interactions. The authors concluded that SNARE proteins play an important role in the fidelity of membrane trafficking and are likely to impart a good deal of the specificity of fusion (Scales *et al.* 2000), but that there are also other proteins which chaperone the formation of the core fusion complex.

1.8.2 The structure of the SNARE complex

Recently the crystal structure of the SNARE complex of syntaxin 1A, synaptobrevin 2 (VAMP2) and SNAP25 has been solved (Sutton *et al.* 1998). Prior to this structure, it was already known that the SNARE proteins form coiled coils (Chapman et al. 1994) and that syntaxin and synaptobrevin lie parallel to each other in the complex (Hanson *et al.* 1997). This is in some ways surprising, because both syntaxin and synaptobrevin/VAMP are anchored in their respective membrane via their C-terminus. On the other hand this position of the SNARE proteins brings the two opposite membranes in close proximity and this might help to drive the fusion of the membranes

(also reviewed in Hughson 1999). The crystal structure revealed that the synaptic fusion complex is arranged as a cylinder consisting of one helix each of syntaxin and synaptobrevin and of two helices of SNAP25 (Figure 1.5). The helices lie such that the N-termini are at one end of the bundle and the C-termini at the other end. SNAP25, which is not an integral membrane protein, but bound to the membrane by palmitylation of cysteine residues present near the middle of the sequence (Chapman et al. 1994), therefore has a linker region between the two helices, which traverses the length of the cylinder. Interestingly, inside the coiled coil there is a hydrophilic layer made of an arginine residue from synaptobrevin and three glutamine residues, one from syntaxin and two from SNAP25 (one from each helix). These residues are thought to stabilise each other and are sealed away from the surrounding solvent by a hydrophobic layer. For this reason synaptobrevin/VAMP is sometimes called an R-SNARE and syntaxin and SNAP25 are called Q-SNAREs. Although it is not yet clear why these polar residues are in the middle of the complex, it was suggested that this could help disassembly of the complex, if the seal was punctured by a protein such as NSF or α-SNAP (Sutton *et al.* 1998).

1.8.3 Syntaxin

Syntaxin was originally discovered by its ability to bind to synaptotagmin/p65 (Bennet *et al.* 1992), a synaptic vesicle protein important in calcium sensing (as reviewed in Südhof 1995). Syntaxin was cloned from rat brain and two isoforms were found, p35A and p35B (syntaxin 1A and 1B) both of which were found to be present largely at the plasma membrane at the site of neurotransmitter release (Bennet *et al.* 1992). Syntaxin is an integral membrane protein, with a short transmembrane region at the C-terminus of

the protein (Bennet *et al.* 1992; Kee *et al.* 1995). The cytoplasmic region of syntaxin contains at least three coiled-coil domains of which the one closed to the transmembrane region (H3) is most important for binding VAMP, α-SNAP and SNAP25 (Calakos *et al.* 1994; Chapman *et al.* 1994; Kee *et al.* 1995; Hanson *et al.* 1995). Mutational studies of syntaxin have shown that the VAMP-syntaxin interaction is very specific and sensitive to mutations, the interaction of SNAP-25 with syntaxin on the other hand is much less specific (Kee *et al.* 1995). Both the H3 region (Wu *et al.* 1999) and the N-terminal helical region of syntaxin 1A can bind synaptotagmin in a Ca^{2+} dependent manner and this is part of the calcium trigger in neurotransmitter release (Fernandez *et al.* 1998). Another protein, important in Ca^{2+} signalling in neuronal cells, that can bind syntaxin on the H3 domain is cysteine string protein (Wu *et al.* 1999) and the authors propose that there is a region in neuronal syntaxin which is important in calcium mediated synaptic vesicle exocytosis.

One protein that is implied to be important in SNARE complex regulation and can bind syntaxin is n-sec1/Munc-18 and will be discussed in more detail below. This protein requires both the amino-terminal domain of syntaxin as well as the H3 domain for binding and prevents binding of syntaxin to the other SNARE proteins (Pevsner *et al.* 1994; Kee *et al.* 1995; Fernandez *et al.* 1998). It was suggested that binding of n-sec1 to syntaxin stabilises intramolecular protein-protein interactions and that this prevents the interaction of other proteins with the H3 region (Kee *et al.* 1995). Interestingly this study also showed that the full cytoplasmic region of syntaxin is needed for dissociation of the 20S complex and it thought that α-SNAP and VAMP with the help of NSF are displaced from the H3 region by the amino-terminal helical regions (H1 and H2) (Kee *et al.* 1995; Hanson *et al.* 1995). It is now thought that syntaxin can adopt at least three different

conformations, one in isolation, another in the SNARE complex and a third when bound to Munc-18 (Misura *et al.* 2000).

As mentioned before, there are many non-neuronal isoforms of syntaxin and syntaxin is not only involved in vesicles fusing with the plasma membrane but also in trafficking between various membrane compartments , such as ER to Golgi and Golgi to endosomes (Bock *et al.* 1996; Advani *et al.* 1998; Prekeris *et al.* 1998; Tang *et al.* 1998). The non-neuronal isoform syntaxin 4 is important in the translocation of GLUT4 and will be discussed in detail below.

1.8.4 VAMP

VAMP1 was originally cloned from the brain of a cartilaginous fish and found to be predominately present on synaptic vesicles (Trimble *et al.* 1988). This protein has a molecular mass of 13kDalton and can be divided into three structural domains. The C-terminus is hydrophobic and spans the vesicle membrane and the N-terminal domain, which contains a proline rich sequence and a long hydrophilic stretch, is exposed to the cytoplasm (Trimble *et al.* 1988). There are many mammalian homologues of VAMP, they vary in length and not all of them have the proline rich N-terminus (Advani *et al.* 1998). As described above, VAMP can bind both syntaxin and SNAP-25 (Söllner *et al.* 1993b) via coiled-coil regions, but the individual interactions between both proteins are relatively weak and it was shown that SNAP-25-syntaxin heterodimers form a high affinity binding site for VAMP (Chapman et al. 1994; Hayashi *et al.* 1994). Unlike syntaxin, VAMP does not bind α-SNAP directly (McMahon & Südhof 1995; Hanson *et al.* 1995) and this suggests a post-docking role of α-SNAP (McMahon & Südhof 1995).

Synaptophysin is a very abundant vesicle membrane protein that forms a high molecular weight complex on the membrane, containing four synaptophysin subunits and one low molecular protein (McMahon *et al.* 1996). This protein has been identified as VAMP and indeed VAMP2 is able to bind synaptophysin via its amino-terminal domain (Washbourne *et al.* 1995). Interestingly though, synaptophysin is not essential for synaptic function as determined by synaptophysin knockout mice (McMahon *et al.* 1996) and its precise role and the importance of the synaptophysin-VAMP interaction remain to be determined.

1.8.5 The regulation of the SNARE complex and Munc18/nsec1

Clearly the regulation of the SNARE complex formation is crucial for the fusion of the correct vesicles with the target membrane. Rab proteins are thought to play a role in regulating the specificity of vesicle trafficking events and the multitude of rab proteins present would make them ideal candidates for SNARE complex regulation (as reviewed in Lin & Scheller 2000). Although rab proteins are not part of the SNARE complex (Söllner *et al.* 1993b), they are thought to play a role in SNARE complex assembly and were suggested to act as a catalyst (Søgaard *et al.* 1994). The rab5 effector EEA1 can bind syntaxin13 transiently (McBride *et al.* 1999). This and other data has suggested that EEA1 is important in tethering endosomal vesicles on the plasma membrane and the subsequent binding of EEA1 to syntaxin could then drive fusion of the membranes (Christoforidis *et al.* 1999; McBride *et al.* 1999; and reviewed in Clague 1998).

Another important regulatory protein of SNARE complex formation is the above mentioned Munc-18. Munc-18/nSec1 is the neuronal homologue of the yeast protein

sec1p and it is known to be important in neurotransmitter release (as reviewed in Lin & Scheller 2000). Munc-18 has been shown to bind syntaxin alone, but not to syntaxin in the SNARE complex, and binding of Munc-18 to syntaxin inhibits the formation of the syntaxin-VAMP-SNAP-25 complex (Pevsner *et al.* 1994). Interestingly phosphorylation of Munc-18 by PKC inhibits the interaction of Munc-18 with syntaxin and this might facilitate the formation of the SNARE complex (Fujita *et al.* 1996). There are several non-neuronal isoforms of Munc-18 that have been found to bind many syntaxin isoforms and which may play a role in different types of exocytosis (Hata & Südhof 1995).

Recently the three-dimensional structure of nSec1/Munc-18 with syntaxin 1A has been solved (Misura *et al.* 2000). Interaction between these two proteins is complex, involving both the N- and C-terminal cytoplasmic domain of syntaxin and the whole of Munc-18. Most interactions between the two proteins are between residues that are conserved in the sequence of the different protein isoforms, but there are also a smaller number of interactions between areas that are not conserved and these are thought to contribute to the specificity between isoforms. One such pair are Munc-18c and syntaxin4 and the importance of Munc-18c in GLUT4 translocation will be discussed below.

Dissociation of the syntaxin-Munc-18 complex is thought to be brought about by a conformational change in Munc-18 and is probably mediated by another protein(s) such as rab or a rab effector. A conformational change in Munc-18 would provide a favourable environment for the SNARE complex formation and it is now known that Munc-18 has not only a negative, but also a positive role in SNARE complex formation, acting as a mediator for the association of these proteins (Misura *et al.* 2000).

1.8.6 The docking and fusion of vesicles with the membrane

The release of neurotransmitter into the presynaptic cleft and indeed many other processes involving the fusion of vesicles with the target membrane, starts with the translocation of those vesicles to the membrane (Figure 1.6). Attachment of the vesicles to the membrane can be divided into tethering and then docking (as reviewed in Foster & Klip 2000). Tethering of the vesicles on the membrane occurs by a so far little known process, probably involving rab proteins and their effectors. Many studies have shown that neither SNARE proteins nor NSF and α-SNAP are required for this step (Hunt *et al.* 1994; DeBello *et al.* 1995; McMahon & Südhof 1995; Nichols *et al.* 1997). The formation of the SNARE complex then docks the vesicles to the membrane and leads ultimately to fusion. Syntaxin starts in the closed conformation bound to Munc-18 as described above. A conformational change in Munc-18, mediated by other proteins, causes the recognition of syntaxin by VAMP and SNAP-25 and subsequent SNARE complex formation. This brings the two membranes into close proximity and leads to fusion by a mechanism not clearly known yet. A recent study suggested that membrane fusion is an active event, and that the SNARE complex exerts force on the membrane anchors, which provides an essential driving force for fusion (McNew *et al.* 2000).

Disassembly of the SNARE complex is known to involve NSF and α-SNAP (Hayashi *et al.* 1995; Nagiec *et al.* 1995; Barnard *et al.* 1997; Rice & Brunger 1999), but in contrast to the initial model, NSF and α-SNAP are not required for fusion itself (Nichols *et al.* 1997). Examining yeast vacuolar fusion has shown that NSF and α-SNAP are involved in a pre-docking stage (Mayer *et al.* 1996). This is surprising, but could be explained by

the suggestion that NSF and α-SNAP are necessary for the disassembly of *cis* SNARE complexes that occur after membrane fusion. In order for a new cycle of vesicle fusion to happen, SNARE proteins have to be disassembled and recycled to their correct location (as reviewed in Lin & Scheller 2000). NSF on the other hand has been found in a complex with rab5 and EEA1 and it was suggested that NSF could prime SNARE proteins before fusion (McBride *et al.* 1999). Clearly more research is needed to determine the full function of all the proteins involved in the translocation and fusion of the vesicles with their target membrane.

1.9 SNARE proteins in GLUT4 translocation

Although GLUT4 exocytosis is a much slower process than neurotransmitter release it resembles this process in many ways. In both mechanisms vesicles containing cargo are stored intracellularly until a signal triggers their translocation to the plasma membrane. For neurotransmitter release this signal is the entry of calcium into the cell, while for GLUT4 exocytosis it is the binding of insulin to its receptor and the subsequent signalling cascade. Docking of vesicles at the plasma membrane then leads to fusion and release of cargo either into the synaptic cleft in neurotransmitter release or by insertion of GLUT4 into the plasma membrane. This similarity led to the search for a SNARE type mechanism in insulin sensitive tissue and resulted in a much more detailed understanding of GLUT4 translocation in these cells (for review see Foster & Klip 2000).

Homologues of many SNARE proteins have been found in fat and muscle cells. VAMP2 and cellubrevin (a VAMP homologue) have been found in rat adipocytes (Cain *et al.* 1992; Martin *et al.* 1996), 3T3-L1 adipocytes (Tamori *et al.* 1996; Martin *et al.* 1996) and muscle cells (Volchuk *et al.* 1994; Kristiansen *et al.* 1996). Functional data using *Clostridial botulinum* neurotoxins have shown that cleavage of cellubrevin and VAMP2 will inhibit insulin-mediated GLUT4 translocation (Cheatham *et al.* 1996; Macaulay *et al.* 1997). Interestingly the cleavage of VAMP2 but not cellubrevin by an IgA protease, also blocked the majority of insulin-stimulated GLUT4 translocation (Cheatham *et al.* 1996). The introduction of GST-VAMP2 into permeabilised adipocytes also inhibited insulin-stimulated GLUT4 translocation (Cheatham *et al.* 1996; Martin *et al.* 1998; Millar *et al.* 1999), whereas by contrast GST-cellubrevin was without effect on insulin-stimulated GLUT4 translocation (Martin *et al.* 1998). GLUT4 translocation was also inhibited by 40%-50% when 3T3-L1 adipocytes were treated with an N-terminal peptide of VAMP2 (Macaulay *et al.* 1997; Olson *et al.* 1997; Martin *et al.* 1998) and this same peptide did not have any effect on insulin-stimulated GLUT1 translocation (Martin *et al.* 1998). On the other hand GST-cellubrevin inhibits insulin-stimulated GLUT1 translocation partially (Millar *et al.* 1999). From this data it was proposed that cellubrevin is important in translocation of endosomal proteins such as GLUT1 to the plasma membrane, while VAMP2 specifically regulates the docking of GSVs in response to insulin.

Much research has focused on the exact intracellular location of VAMP2 and cellubrevin. Both proteins translocate from intracellular stores to the plasma membrane in response to insulin and are found in GLUT4 vesicles (Cain *et al.* 1992; Martin *et al.* 1996; Tamori *et al.* 1996; Timmers *et al.* 1996). Endosome ablation showed that

cellubrevin was ablated to a similar degree as TfR, while VAMP2 was only ablated by about 10%. This data and vesicle adsorption together with ablation suggest that cellubrevin is enriched in the endosomal GLUT4 vesicles, while VAMP2 is present in the GSVs (Martin *et al.* 1996).

Syntaxin4 is expressed in many tissues and high levels of it were found at the plasma membrane in adipocytes (Timmers *et al.* 1996; Tellam *et al.* 1997) and muscle (Volchuk *et al.* 1996). Low levels of syntaxin4 were found in intracellular membranes (Timmers *et al.* 1996; Tellam *et al.* 1997) and from there it appears to translocate to the plasma membrane in response to insulin in a similar manner as GLUT4 (Tellam *et al.* 1997), the functional significance of this is not clear.

Insulin stimulated glucose transport was shown to be inhibited by the injection of either antibodies to syntaxin4 (Tellam *et al.* 1997) or of a syntaxin4 peptide into adipocytes (Macaulay *et al.* 1997; Olson *et al.* 1997). Similarly the treatment of permeabilised 3T3-L1 adipocytes with GST-syntaxin4 caused the inhibition of insulin-stimulated GLUT4 translocation (Cheatham *et al.* 1996). Interestingly the introduction of a cytoplasmic syntaxin4 peptide into cells did not inhibit insulin-mediated GLUT1 translocation (Olson *et al.* 1997). This set of data supports the specific role for syntaxin4 in GLUT4 vesicle fusion at the plasma membrane.

Evidence for the presence of SNAP25 in adipocytes is ambiguous (Jagadish *et al.* 1996; Wong *et al.* 1997), but two SNAP25 homologues called SNAP23 and syndet have been found to be expressed in fat and muscle tissue (Ravichandran *et al.* 1996; Wong *et al.* 1997; Araki *et al.* 1997; Rea *et al.* 1998). Both proteins are as expected predominantly

located to the plasma membrane fraction (Araki *et al.* 1997; Wong *et al.* 1997; Rea *et al.* 1998).

An antibody to syndet as well as a C-terminal peptide have been shown to inhibit insulin stimulated GLUT4 translocation by 40%, but did not inhibit GLUT1 translocation (Rea *et al.* 1998). Binding studies using yeast two hybrid have revealed that SNAP23 can bind strongly to syntaxin1 and 4 (Araki *et al.* 1997) as well as VAMP1 and 2 (Ravichandran *et al.* 1996). Similarly, surface plasmon resonance experiments have shown that syndet can bind syntaxin4 and VAMP2 individually and that this is disrupted by 0.5% SDS, while incubation of all three proteins together resulted in a 0.5% SDS resistant complex formation (Rea *et al.* 1998).

Screening of rat adipocytes for NSF and α-SNAP revealed that both proteins are intracellularly located and to a substantial extent are present on GLUT4 vesicles (Mastick *et al.* 1997).

It is now thought that in insulin-sensitive tissues GLUT4 translocation occurs via a SNARE type mechanism similar to neuronal cells involving the formation of a complex made of syntaxin4, VAMP2, syndet/SNAP23 as well as NSF and α-SNAP. Indeed the formation of a 20S SNARE complex in vitro has been confirmed and shown to be independent of insulin treatment (St-Denis *et al.* 1999).

The importance of a SNARE-mechanism for GLUT4 translocation leads to the question of how this is regulated and how insulin effects this mechanism. As mentioned above, there are several proteins important for the regulation of the SNARE complex. One such

candidate is Munc-18 of which several isoforms exist (Tellam *et al.* 1995). Munc-18c is ubiquitously expressed and its subcellular location in adipocytes is almost identical to that of syntaxin4 with highest levels found in the plasma membrane fraction and lower levels in the LDM fraction (Tellam *et al.* 1997). In vitro binding studies showed that Munc-18c bound strongly to syntaxin4 (Tellam *et al.* 1997; Tamori *et al.* 1998) and that it was able to reduce the association of syntaxin4 and VAMP2 by 75%. Similarly Munc-18c was also able to reduce the binding of cellubrevin to syntaxin4 by 60% (Tellam *et al.* 1997). Co-immunoprecipitation experiments showed that syntaxin4 can bind both SNAP23 and Munc-18c *in vivo*, but that Munc-18c decreases the affinity of syntaxin4 for SNAP23 (Araki *et al.* 1997). Overexpression of Munc-18c in 3T3-L1 adipocytes inhibits insulin-stimulated GLUT4, but not GLUT1 translocation (Tamori *et al.* 1998). Similarly a Munc-18c peptide can inhibit GLUT4 and IRAP integration into the plasma membrane (Thurmond *et al.* 2000). In these cells GLUT4 vesicles were found to be located just below the membrane unable to fuse with the plasma membrane, probably due to the peptide binding to all the available syntaxin4, thereby preventing the formation of the SNARE complex (Thurmond *et al.* 2000).

Another SNARE complex regulator is synip (Min *et al.* 1999). Synip is a syntaxin4 binding protein and its expression is restricted to adipocyte and muscle cells. Interestingly in the presence of insulin the affinity of synip for syntaxin4 is reduced suggesting that this protein is an insulin-sensitive regulator for GLUT4 translocation. This is supported by the fact that a C-terminal peptide of synip can inhibit insulin-mediated GLUT4 but not GLUT1 translocation. It is thought that the N-terminus of synip contains a regulatory domain, which causes the insulin-mediated decrease of

syntaxin4-synip interaction, thereby allowing SNARE complex formation and subsequent fusion of the GSVs with the plasma membrane (reviewed in Bennett 1999).

The question of how insulin regulates the formation of the SNARE complex and ultimately fusion of the GLUT4 vesicles with the plasma membrane cannot be totally explained by synip. As above mentioned, there are other proteins such as the small GTPase rab which might control the SNARE complex formation. Indeed a direct interaction of syntaxin4 with rab4 has been shown recently and this interaction was regulated by the guanine nucleotide status of rab4 and therefore indirectly by insulin (Li *et al.* 2001). The significance of this interaction was not determined and so far there is little known about what function rab proteins have in insulin-stimulated glucose transport.

Other proteins that have recently been identified to interact with SNARE proteins in insulin-sensitive cells are pantophysin and cysteine string protein (Brooks *et al.* 2000; Chamberlain *et al.* 2001). The cysteine-string protein Csp1 interacts with syntaxin4 in 3T3-L1 adipocytes and it was proposed that this protein might be a chaperone of syntaxin4, regulating the conformational status of this t-SNARE (Chamberlain *et al.* 2001). Pantophysin is homologous to synaptophysin and can interact with VAMP2, but the significance of this is not yet known (Brooks *et al.* 2000).

From these studies it is clear that the SNARE mechanism is absolutely vital for GLUT4 exocytosis into the plasma membrane and subsequent glucose transport. Decreased insulin-stimulated glucose uptake due to a defect in GLUT4 translocation could therefore be explained by a fault in the SNARE mechanism. So far there are no studies

looking at SNARE proteins in insulin resistance and diabetes and in this thesis we therefore set out to examined SNARE levels and distributions in an insulin resistant model system (Chapter 4).

1.10 Aims of this Study

The objective of this thesis was to further characterise the GLUT4 trafficking pathways both under normal and insulin resistant conditions. Therefore proteins which are important in GLUT4 targeting and translocation were studied.

In Chapter 3 we look at a new method using iodixanol as a gradient material to separate the two intracellular GLUT4 compartments and also investigate GSV formation during differentiation.

Insulin resistance precedes the development of diabetes and in order to learn more about the cellular mechanism which causes this we studied insulin resistant 3T3-L1 adipocytes in Chapter 4.

In Chapter 5 we tried to characterise the adaptor proteins in adipocytes and examined whether there are any direct interactions between these proteins and GLUT4.

Figure 1.1 PI3-kinase is a central enzyme in insulin mediated glucose uptake

The insulin receptor uses IRS proteins to activate PI3-kinase. Activated insulin receptor binds transiently to IRS, which becomes phosphorylated. Phosphorylated IRS binds the regulatory subunit (p85) of PI3-kinase and this will activate the catalytic subunit (p110). The IRS/PI3-kinase complex translocates to internal membranes (LDM), which contain the GLUT4 vesicle pool as well as microtubules. In response to insulin GLUT4 translocates to the plasma membrane (PM).

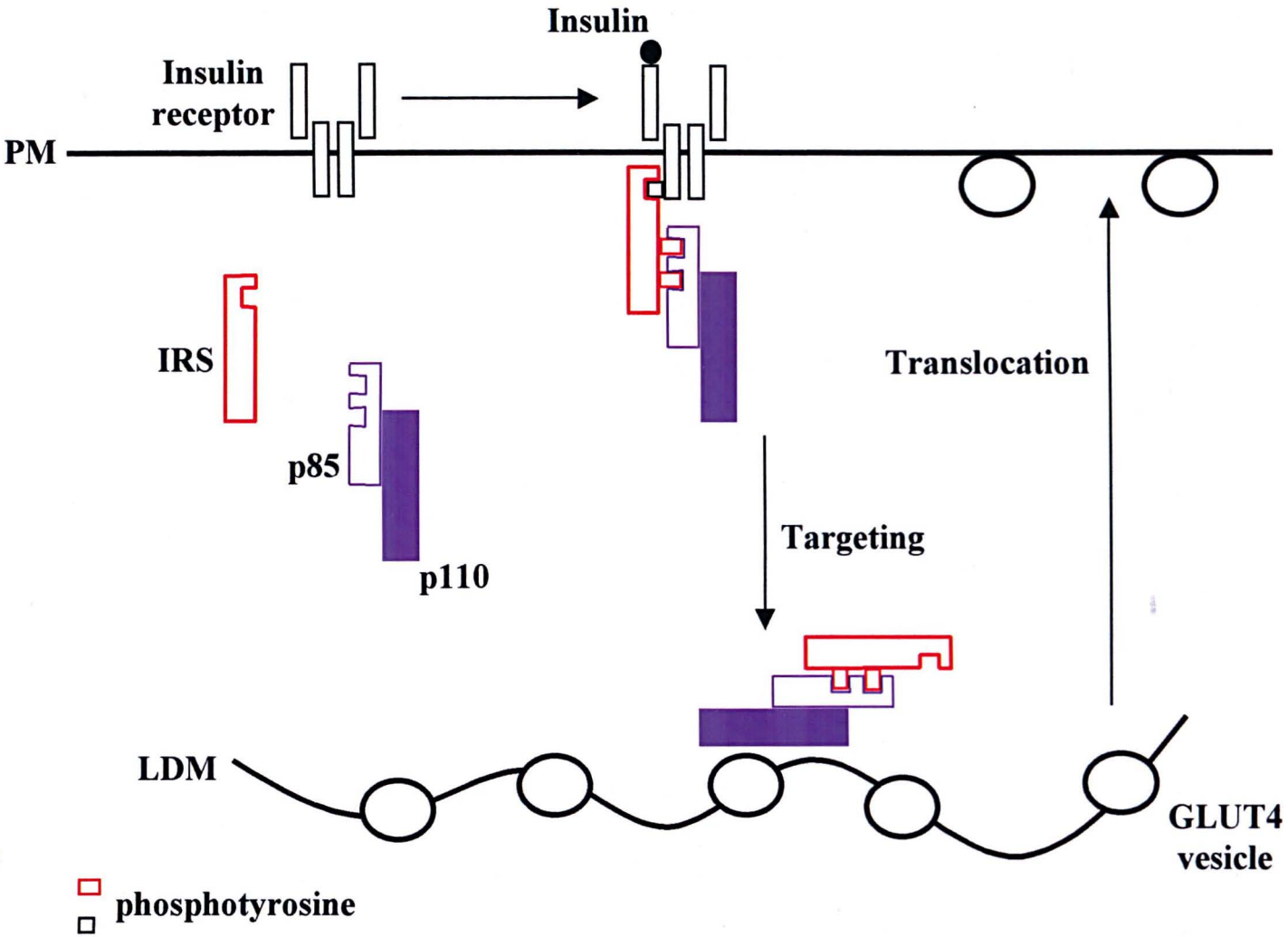

Insulin
Insulin receptor
PM
IRS
p85
p110
Translocation
Targeting
LDM
GLUT4 vesicle
phosphotyrosine

Figure 1.1

Figure 1.2 Glucose transporter structure

A schematic model for the transmembrane organisation of a GLUT. Shown are the 12 membrane spanning helices and the large loops between helices 1 and 2 and 6 and 7. Note that both N- and C-terminus are intracellular.

Figure 1.2

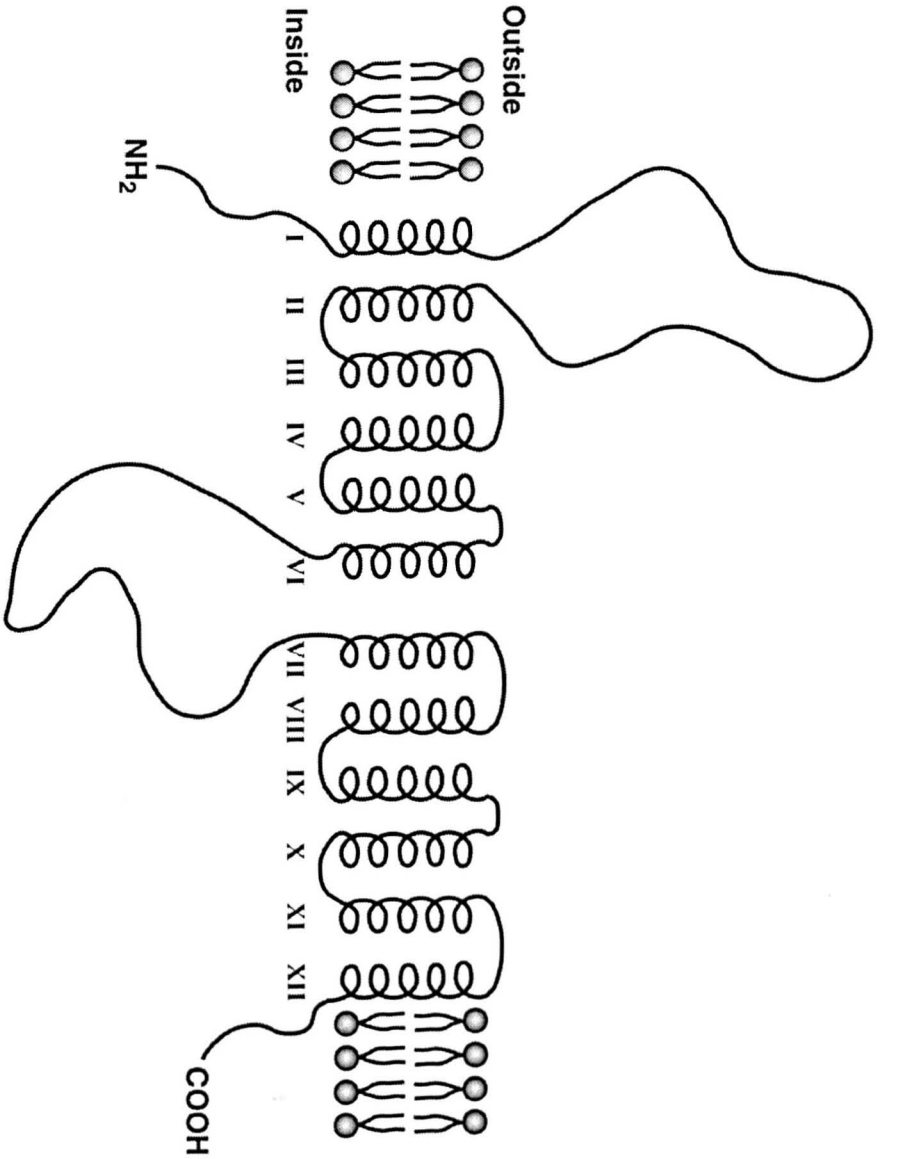


Figure 1.3 Membrane protein recycling models (Holman *et al.* 1994)

A In the 2-pool model, the fully functional plasma membrane protein (T_p) is in equilibrium with only one intracellular pool (T_{ee}). **B** In the 3-pool model, two distinct intracellular pools are designated, the early endosomal pool (T_{ee}). and the tubulo-vesicular compartment (T_{tv}). **C** and **D** In the 4-pool models, occluded forms are added to the plasma membrane occurring either before (T_{po}, **C**) or after (T_{pc}, **D**) the fully functional plasma membrane pool. **E** In the 5-pool model, two occluded plasma membrane pools are included as well as two intracellular pools.

Figure 1.3

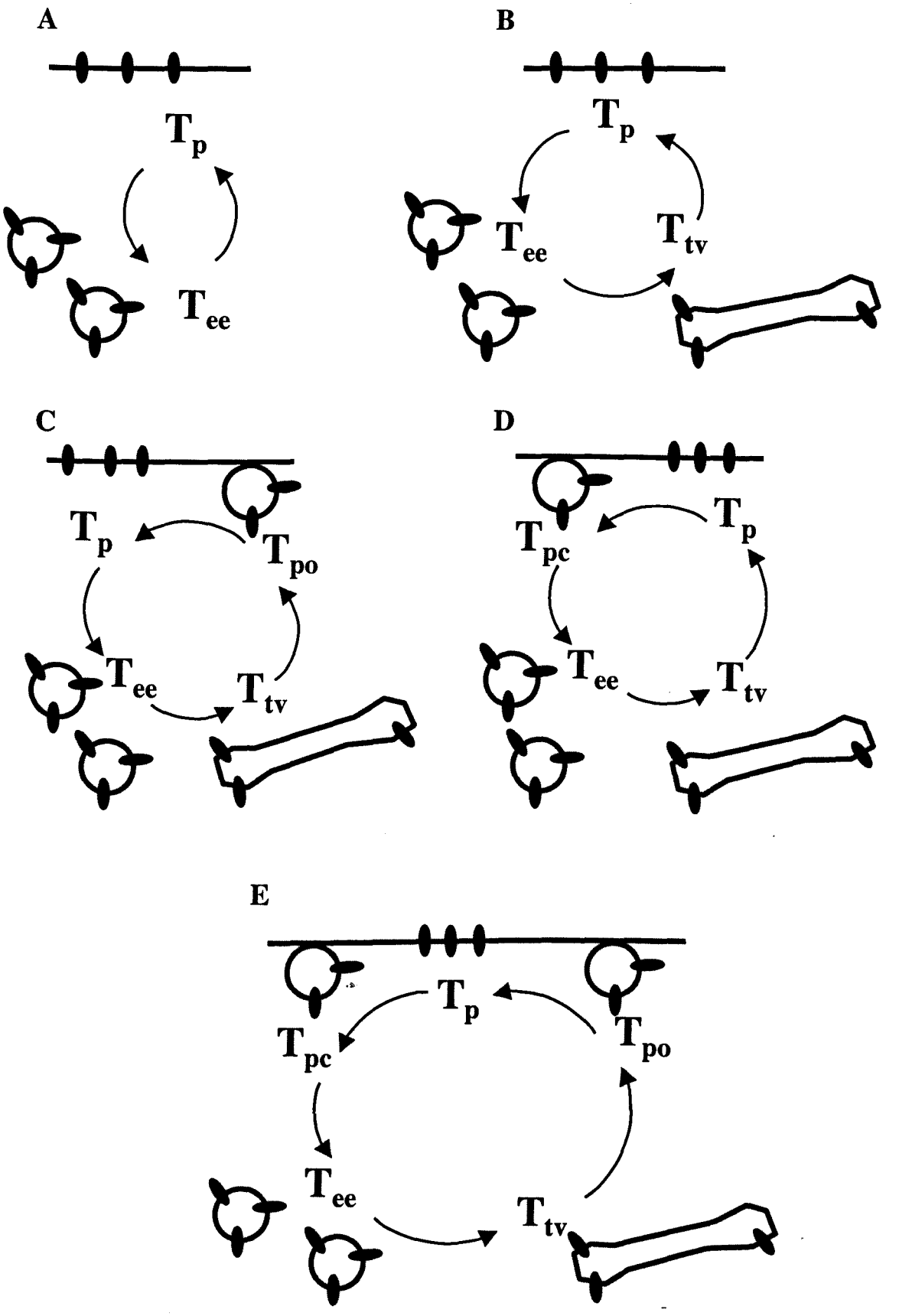

A
T_p
T_{ee}
B
T_p
T_{ee}
T_{tv}
C
T_p
T_{po}
T_{ee}
T_{tv}
D
T_{pc}
T_p
T_{ee}
T_{tv}
E
T_p
T_{pc}
T_{po}
T_{ee}
T_{tv}

Figure 1.4 GLUT4 trafficking in cells

Shown is a schematic representation of both GLUT4 and transferrin receptor trafficking. Both proteins are internalised from the plasma membrane (PM) into endosomes, from where they are trafficked into sorting endosomes. Most of the transferrin receptor, but a low percentage of GLUT4 then recycle back to the plasma membrane. GLUT4 is packed into specialised GLUT4 storage vesicles (GSVs); this step could arise directly from endosomes, or from the trans-Golgi network (TGN). Insulin causes the fusion of the GSVs with the plasma membrane and therefore the insertion of GLUT4 into the plasma membrane.

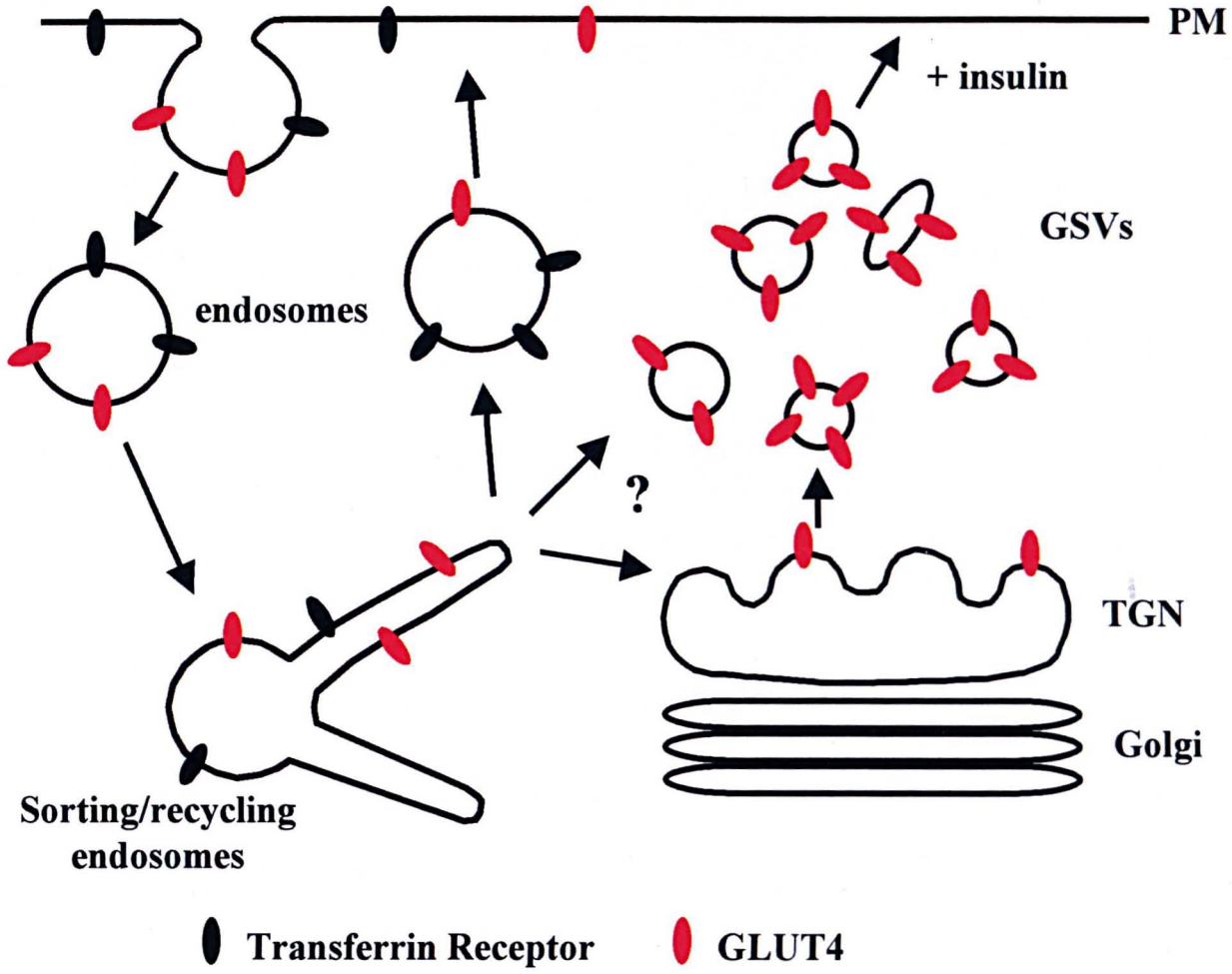

PM
+ insulin
GSVs
endosomes
?
TGN
Golgi
Sorting/recycling
endosomes
Transferrin Receptor
GLUT4

Figure 1.4

Figure 1.5 Hypothetical model of the synaptic fusion complex (Sutton *et al.* 1998)

Four helices, one from syntaxin (red), one from VAMP (blue) and two from SNAP-25 (green), form a coiled coil structure. The transmembrane domains of VAMP and syntaxin are included (yellow) as well as the linker region of SNAP-25 (brown). Note that the here illustrated helices represent only a part of each protein.

Figure 1.5

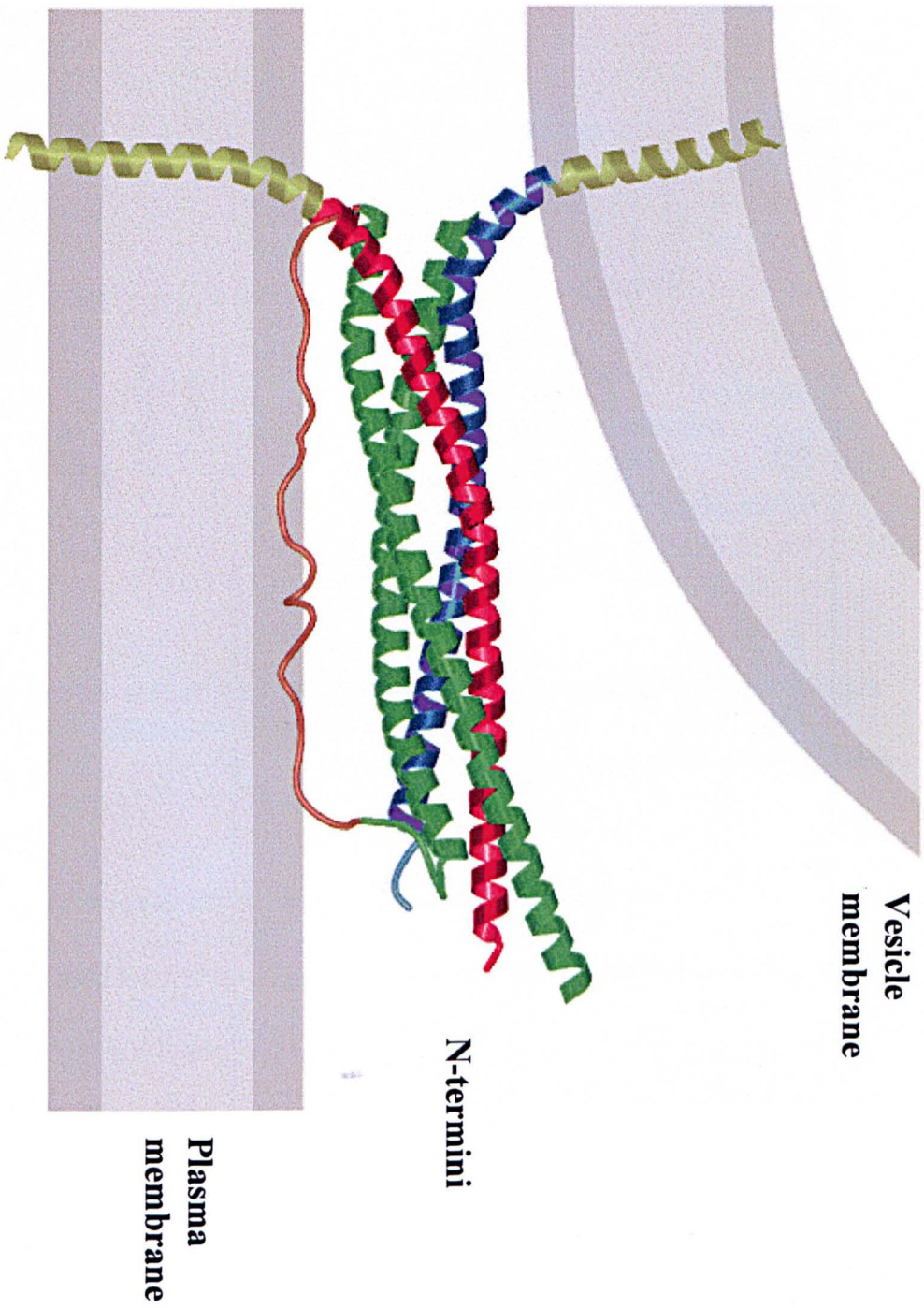


Figure 1.6 Simplified model for the docking and fusion of the vesicles with the membrane

Vesicles filled with neurotransmitter translocate to the plasma membrane (PM). NSF and α-SNAP may have a role in vesicle priming. Vesicles are then tethered to the target membrane via a little known process, possibly involving rab proteins and their effectors (see text for detail). After the release of syntaxin by Munc-18, the SNARE complex assembles and drives membrane fusion. *Cis*-SNARE complexes are then separated with the help of NSF and α-SNAP. Note that the role of NSF in this event is still unclear. Before the next vesicles can fuse with the membrane, VAMP has to be recycled into its correct intracellular compartment.

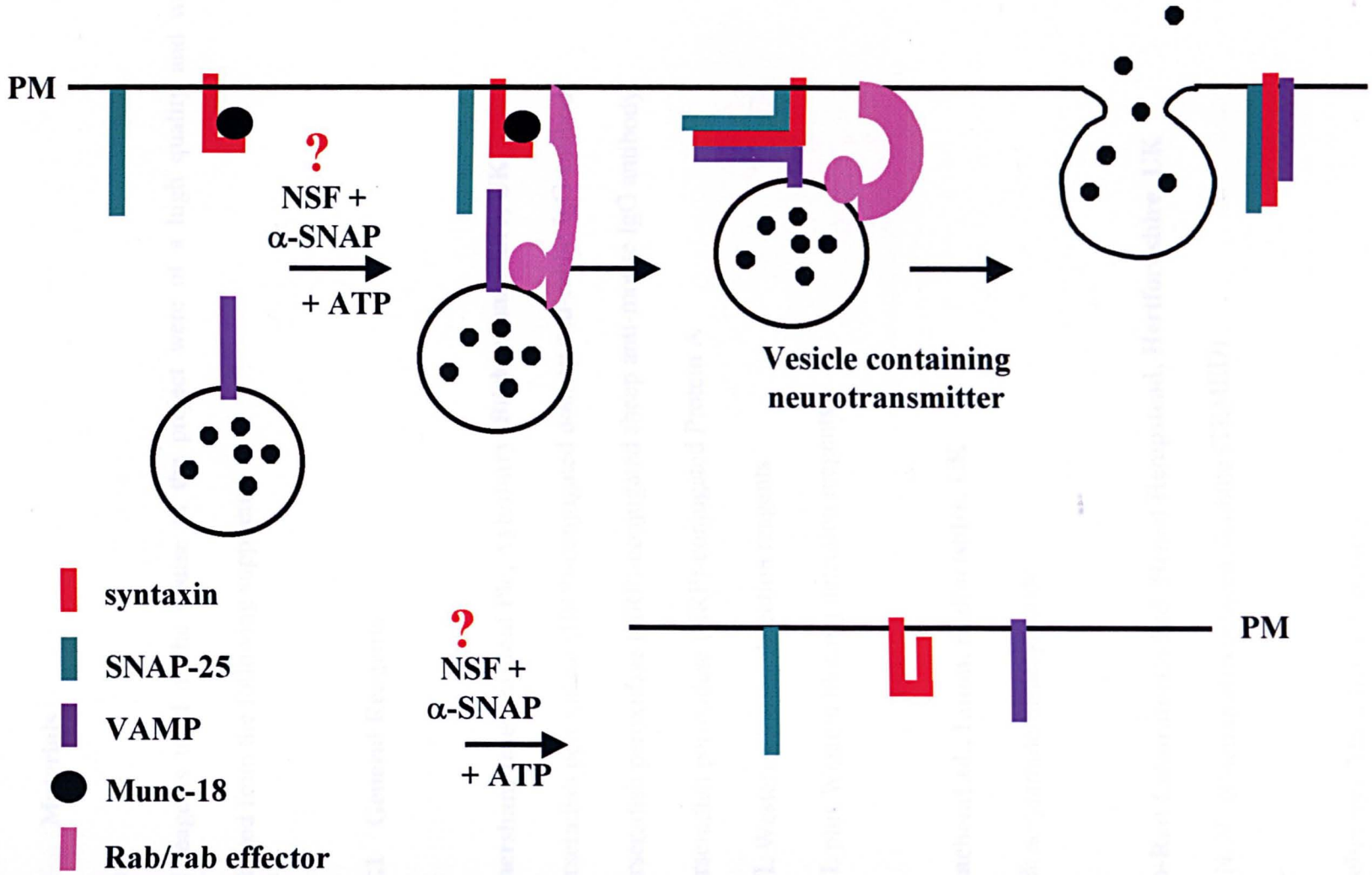

PM
?
NSF +
α-SNAP
+ ATP
Vesicle containing
neurotransmitter
syntaxin
SNAP-25
VAMP
Munc-18
Rab/rab effector
?
NSF +
α-SNAP
+ ATP
PM

Figure 1.6

2.1 Materials

All reagents used in the course of the project were of a high quality and were obtained from the following suppliers:

2.1.1 General Reagents

Amersham International Plc, Aylesbury, Buckinghamshire, UK

Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody

Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody

Horseradish peroxidase (HRP)-conjugated Protein A

ECL Western blotting detection reagents

ECL plus Western blotting detection reagents

AnachemLtd., Luton, Bedfortshire, UK

30% acrylamide/bisacrylamide

Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK

N, N, N', N'-tetramethylethylenediamine (TEMED)

Boehringer Mannheim, Germany

Chlorophenol red-β-D-galactopyranoside (CPRG)

Tris

Protease Inhibitor cocktail tablets, complete™

Protease Inhibitor cocktail tablets, complete™, Mini

Difco Laboratories, Detroit, USA

Yeast nitrogen base (without amino acids)

Fisher Ltd., Loughborough, Leicestershire, UK

Ammonium persulphate

Ammonium sulfate

Boric acid

Calcium Chloride ($CaCl_2$)

Diaminoethanetetra-acetic acid, Disodium salt (EDTA)

Disodium hydrogen orthophosphate (Na_2HPO_4)

Glucose

Glycerol

Glycine

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

Hydrochloric acid (HCl)

Magnesium sulfate ($MgSO_4$)

Methanol

Potassium chloride (KCl)

Potassium Dihydrogen orthophosphate (KH_2PO_4)

Sodium dodecyl sulphate (SDS)

Sodium chloride (NaCl)

Sodium dihydrogen orthophosphate dihydrate (NaH_2PO_4)

Sodium hydrogen carbonate ($NaHCO_3$)

Sucrose

Trichloroacetic acid

Tri-Sodium citrate ($Na_3C_6H_5O_7$)

Kodak Ltd, Hemel Hempstead, Hertfordshire, UK

X-Omat S film

Merck Ltd (BDH), Lutterworth, Leicestershire, UK

Ethanolamine

Magnesium chloride ($MgCl_2$)

Tween 20

Zinccloride ($ZnCl_2$)

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

[^{125}I] transferrin

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

Prestained protein marker, broad range (6-175kDa)

Novo Nordisk, Denmark

Insulin (Porcine)

Nycomed, Norway

OptiPrep™ (Iodixanol)

Oxoid Ltd, Hampshire, UK

Bacteriological agar

YEPD

Pierce, Rockford, Illinois, USA

BCA™ Protein Assay Kit

Premier Brands UK, Knighton Adbaston, Staffordshire, UK

Marvel powdered milk

Schleicher & Schuell, Dassel, Germany

Nitrocellulose membrane (pore size:0.45μM)

Whatman International Ltd, Maidstone, UK

Whatman No.1 filter paper

Whatman No.3 filter paper

Whatman 3mm filter paper

2.1.2 Cell Culture Materials

American Type Culture Collection, Rockville, USA

3T3-L1 fibroblasts

Gibco BRL, Paisley, Lanarkshire, UK

Foetal bovine serum (FBS)

New born calf serum (NCS)

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

10000U/ml penicillin, 10000U/ml streptomycin

Trypsin/EDTA solution

AS Nunc, DK Roskilde, Denmark

50 ml centrifuge tubes

Costar

6 cm cell culture plates

75cm^2 cell culture flasks

Falcon

10 cm cell culture plates

6-well cell culture plates

Bibby Sterilin Ltd, Stone, Staffordshire, UK

Sterile pipettes

13.5 ml centrifuge tubes

Unless otherwise indicated, all remaining chemicals were supplied by Sigma Chemical Company Ltd, Poole, Dorset, UK.

2.1.3 Antibodies

GLUT4 antibody was raised in rabbit and affinity purified

Transferrin antibody was from Zymed Laboratories, Cambridge, UK

AP2 antibody was from Sigma, Chemical Company Ltd, Poole, Dorset, UK.

Syndet/SNAP-23 antibody was from Synaptic Systems, Göttingen, Germany

The IRAP antibody was monoclonal and kindly provided by Morris Birnbaum, University of Pensylvania.

Following antibodies were the kind gift of David James, Australia: VAMP2, syntaxin4 and cellubrevin.

Following antibodies were kindly provided by Margret Robinson, Cambridge, UK: AP1 (γ), AP3 (δ and μ) and AP4 (β and ε).

TGN 38 antibody was kindly provided by George Banting, Bristol, UK.

Pacs-1 antibody was kindly provided by Dr Yang, USA.

AP3 (δ) was a kind gift of Jim-Tong Horng, University of California, San Francisco USA.

2.2. General Buffers

2.2.1 Phosphate Buffered Saline (PBS)

136mM NaCl, 10mM NaH_2PO_4, 2.5mM KCl, 1.8mM KH_2PO_4, pH 7.4.

2.2.2 Krebs Ringer Phosphate (KRP) Buffer

128mM NaCl, 4.7mM KCl, 5mM NaH_2PO_4, 1.25mM $MgSO_4$, 1.25mM $CaCl_2$, pH 7.4.

2.2.3 HES Buffer

255mM sucrose, 20mM HEPES, 1mM EDTA, pH 7.4.

2.2.4 Krebs Ringer MES (KRMES) Buffer

136mM NaCl, 4.7mM KCl, 1.25mM $CaCl_2$, 1.25mM $MgSO_4$, 10mM MES, 25mM Glucose, pH 6.

2.2.5 Kreps Ringer HEPES (KRHEPES) Buffer

136mM NaCl, 4.7mM KCl, 1.25mM $CaCl_2$, 1.25mM $MgSO_4$, 10mM HEPES, pH 7.4.

2.2.6 Lysis/Immunoprecipitation Buffer

50mM HEPES, 1mM EDTA, 10mM $MgCl_2$, 150mM NaCl, 1mM sodium orthovanadate, 10% Glycerol, 1% Triton, pH 7.4

2.3 Cell Culture

2.3.1 3T3-L1 Murine Fibroblasts

3T3-L1 fibroblasts were cultured in 75cm^2 flasks containing DMEM/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% CO_2 and the media was replaced every 48 h. When subconfluent (showing 80% confluency) the cells were passaged into 6-well or 10cm culture plates and a 75cm^2 carry over flask. Cells were cultured to 4 days post confluency and then differentiated into adipocytes.

2.3.2 Trypsinisation and Passage of 3T3-L1 Fibroblasts

Following aspiration of media from a 75cm^2 flask, 1ml of trypsin/EDTA solution was added to wash the remaining media from the monolayer of cells. In turn, this was aspirated and 3ml of trypsin/EDTA solution was added and the flask was incubated at 37°C in a humidified atmosphere of 10% CO_2 for 5 min. Careful agitation of the flask resulted in the cells being lifted from the surface of the flask. This cell suspension was then added to a maximum volume of 230ml DMEM/10% (v/v) newborn calf serum and 1% (v/v) penicillin and streptomycin. With occasional agitation the cells were split between 10cm culture plates and /or 6-well culture plates and 2 x 75cm^2 flasks.

2.3.3 Differentiation of 3T3-L1 Fibroblasts

Differentiation media of DMEM/10% (v/v) foetal bovine serum, 1% (v/v) penicillin and streptomycin, 0.25μM dexamethasone, 0.5mM methyl isobutylxanthine and 1μg/ml insulin was prepared as outlined below.

A stock of 2.5mM dexamethasone (in ethanol) was diluted 1:20 with DMEM/ 10% (v/v) foetal bovine serum, 1% (v/v) penicillin and streptomycin immediately prior to use yielding a 500X stock solution. A 500X sterile solution of methyl isobutylxanthine (IBMX) was prepared by dissolving 55.6mg IBMX in 1ml of 2M KOH and passing the solution through a 0.22 micron filter. Insulin (1mg/ml) was prepared in 0.01M HCL and filter sterilised using a 0.22 micron filter as before. Differentiation media was prepared by adding both the dexamethasone and IBMX solutions to a 1X concentration in DMEM/10% (v/v) foetal bovine serum, 1% (v/v) penicillin and streptomycin and then adding insulin to a final concentration of 1μg/ml.

3T3-L1 fibroblasts were cultured on 6-well or 10cm culture plates until 4 days post confluency. The growth media was aspirated and replaced with differentiation media. After 48 h, this media was replaced with DMEM/10% (v/v) foetal bovine serum, 1% (v/v) penicillin and streptomycin and insulin at 1μg/ml. The cells were incubated in this media for a further 48 h. Following differentiation, the media was replaced with DMEM/10% (v/v) foetal bovine serum, 1% (v/v) penicillin and streptomycin. The media was changed every 48 h and the cells were used 10-14 days post differentiation.

2.3.4 Freezing and Storage of Cells

Fibroblasts were cultured to 80% confluency, the media was aspirated and 1ml of trypsin/EDTA solution was added to wash the remaining media from the monolayer of cells. In turn, this was aspirated and 3ml of trypsin/EDTA solution was added and the flask was incubated at 37°C in a humidified atmosphere of 10% CO_2 for 5 min. Careful agitation resulted in the cells being lifted from the surface of the flask. 3ml of DMEM/10% (v/v) newborn calf serum, 1% (v/v) penicillin and streptomycin was added to the cell suspension and gently triturated over the surface of the flask. The cell suspension was then transferred to a sterile tube and centrifuged at 2000 x g for 4 min. Following aspiration of the supernatant the pellet was 'flicked' gently and then resuspended in 1ml of DMEM/10% (v/v) newborn calf serum, 1% (v/v) penicillin and streptomycin and 10% (v/v) DMSO. The suspension was then transferred to a 1.8ml polypropylene cryo-vial and placed at -80°C overnight to freeze slowly before being stored in a liquid nitrogen vat.

2.3.5 Resurrection of Frozen Cell Stocks from Liquid Nitrogen

A vial of cells was removed from from liquid nitrogen, transferred to a 37°C water bath and thawed. The cell suspension was then pipetted into a 75cm^2 culture flask containing DMEM/10% (v/v) newborn calf serum, 1% (v/v) penicillin and streptomycin which had previously been equilibrated at 37°C in a humidified atmosphere of 10% CO_2. The media was replaced after 24 h and the cells were cultured as described before.

2.3.6 Collagen coating of Cell Culture Plastic Ware

Collagen coated plates were often used for permeabilisation experiments and PM lawn assays to enhance adhesion of the cells to the plate surface upon differentiation. A solution of collagen in acetic acid was used to wash the plates leaving a thin film coating after aspiration. Plates were then dried in a sterile flow hood and irradiated with U.V light overnight. Prior to use, plates were washed with serum-free DMEM to remove any traces of acetic acid.

2.4 Antibody Preparations

The anti-GLUT4 antibody used was rabbit polyclonal antibody against a peptide comprising the C-terminal 14 amino acid residues of the human isoform of GLUT4 described in Brant *et al.* 1993.

2.4.1 Purification of Anti-GLUT4 Antibody

This procedure required the use of an affinity column consisting of Separose beads to which the antigenic peptide (C-terminal 14 amino acid residues) of the human GLUT4 isoform was covalently attached.

Following equilibration of the column with approximately 20ml of PBS (see Section 2.2.1) 300µl of 10X PBS was added to 2.7ml antisera (raised against the GLUT4 C-terminal peptide) and applied to the column. The column was incubated for 30 min at room temperature and then washed with PBS until all the brown coloured eluate

had been collected. The eluant was reapplied and again incubated in the column for 30 min at room temperature. Following the final incubation, the column was washed with PBS, until the eluant gave an OD reading at 280nm of zero. The GLUT4 antibody was eluted with 2M glycine, pH 2, collected in 1ml fractions and the OD reading at 280nm determined for each fraction. The fractions with the highest OD readings were pooled and 50µl 1M Tris was added per 1ml antibody in order to adjust the pH. This was dialysed overnight against PBS, at 4°C and finally, aliquoted, snap frozen and stored at -20°C.

2.5 Protein Assay

Protein concentration was measured using the BCA assay with all samples being performed in duplicate. A standard curve, using 10µl of prepared standards (0.2, 0.4, 0.6, 0.8, 1, 1.5 and 2mg/ml BSA) was set up in a 96-well plate. In general 10µl of samples were used for analysis. BCA buffers were mixed according to the manufacturer's instructions and 200µl of this solution were added to each well. The 96-well plate was covered with clingfilm and incubated at 37°C for 30 minutes. After this time absorbance was measured at 500nm in a plate reader (LKB Wallace, Helsinki, Finnland). A standard curve was plotted and the concentrations of the samples was determined.

2.6 SDS/Polyacrylamide Gel Electrophoresis

SDS/polyacrylamide gel electrophoresis was carried out using Bio-Rad mini-PROTEAN II or Hoefer large gel apparatus. The percentage of acrylamide in each gel ranged between 7.5% - 15%, according to the molecular weight of the protein of interest. All reagents were of electrophoresis grade.

On assembly of either Bio-Rad mini-PROTEAN II or Hoefer large gel apparatus, the seperating gel was prepared using 30% acrylamide/bisacrylamide, 1.5M Tris-HCL (pH 8.8) (to a final concentration of 375mM), 10% (w/v) SDS (to a final concentration of 0.1%), polymerised with 10% (w/v) ammonium persulphate (to a final concentration of 0.1%) and TEMED (to a final concentration of 0.019%). The stacking gel was prepared using 30% acrylamide/bisacrylamide, 1M Tris-HCL (pH 6.8) (to a final concentration of 125mM), 10% (w/v) SDS (to a final concentration of 0.1%), polymerised with 10% (w/v) ammonium persulphate (to a final concentration of 0.1%) and TEMED (to a final concentration of 0.05%).

Protein samples were resuspended in 1X SDS PAGE sample buffer (93mM Tris-Cl pH6.8, 20mM dithiothreitol [added immediately before use], 1mM sodium EDTA, 10% (w/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) and loaded onto the wells in the stacking gel. Broad-range pre-stained molecular weight markers [Mr 6-175 kDa (Biolabs)] were used routinely. Gels were electrophoresed in electrode buffer (25mM Tris, 190mM glycine and 0.1% (w/v) SDS) at a constant voltage to ensure good separation of the molecular weight markers.

2.7 SDS/Polyacrylamide Gel Staining

2.7.1 Coomassie blue staining of protein gel

Following SDS/Polyacrylamide Gel Electrophoresis a 0.25% Coomassie blue stain solution was prepared. For this 10ml of glacial acetic acid was mixed with 90ml of a methanol:H_2O (1:1 v/v) mixture and 0.25g of Brilliant Blue R were added. This stain was then filtered through a Whatman number1 filter paper. The gel was submerged in the stain for about 2h and destained with the same acetic acid, methanol:H_2O mixture (without the dye) as described above. Destaining was for at least 3h or O/N, depending on the resolution wanted. To record the results the gel was photographed.

2.7.2 Silver Staining

Following SDS/Polyacrylamide Gel Electrophoresis, the gel was submerged in 50% methanol and left in this to fix for at least 2h. Just before the staining 800mg of silver nitrate ($AgNO_3$) were dissolved in 4ml of H_2O. Then, in a separate beaker, 2.1ml of NH_3 (14.1M) were added to 21ml of 0.36% NaOH and to this mixture the silver nitrate solution was added dropwise. This was then filled up to 100ml with H_2O. The fixed gel was then submerged in this solution and gently rocked for 5min. The gel was then washed several times in H_2O over a time-period of 15min. In order to develop the stain 250µl of 1% citric acid and 250µl of 38% formaldehyde were added to 500ml of H_2O and the gel was submerged in this and again gently rocked. The gel was observed all the time and the solution was taken off and replaced with water once the protein bands appeard (about 2min). The gel was then again washed several

times with H_2O and stored in 50% methanol. In order to record the results the gel was photographed.

2.8 Western blotting of Proteins

Proteins were separated by SDS/polyacrylamide gel electrophoresis as previously described. A sponge pad, two sheets of Whatman 3mm filter paper and a sheet of nitrocellulose membrane (pore size 0.45mM), cut to size and previously soaked with transfer buffer (25mM NaH_2PO_4, pH 6.5) were placed on the black plate of the gel cassette holder. The gel was then placed on top of the nitrocellulose membrane and any air bubbles were gently removed. The "sandwich" was completed with two more sheets of filter paper and a sponge pad as before. Transfer was carried out using a Bio-Rad mini or Bio-Rad trans-blot electrophoretic cell containing transfer buffer at a constant current of 250mA for 3 h or overnight at 40mA. The efficiency of transfer was determined by staining the nitrocellulose with Ponceau S solution prior to blocking.

2.9 Immunodetection of Proteins

2.9.1 Incubation with antibodies

Following transfer, non-specific binding sites on the nitrocellulose membrane were blocked by shaking for at least 1 h in 5% (w/v) dried skimmed milk (see section 2.1.1) in TBST-1 buffer (20mM Tris, 150mM NaCl, 0.02% Tween-20, pH 7.4).

The nitrocellose membrane was then transferred to a plastic pocket containing primary antibody in 1% (w/v) dried skimmed milk in TBST-1 buffer at the appropriate dilution and shaken for at least 2 h at room temperature. Following five washes with TBST-1 buffer over a period of 1 h, the membrane was incubated in 1% (w/v) dried skimmed milk in TBST-1 buffer with the appropriate secondary antibody, (HRP-linked IgG) at a 1:1000 dilution for 1 h at room temperature. The membrane was then washed a further five times over a period of 1 h.

2.9.2 Using the ECL Detection System

Following incubation with the appropriate HRP-linked IgG and subsequent washing, the membrane was washed in distilled H_2O. The signal was detected using either ECL or ECL plus. For ECL, equal volumes of Amersham "detection reagent 1" and "detection reagent 2" were mixed and the membrane was immersed and shaken in this mixture for 60 s. For ECL plus, Amersham detection reagent A and B were mixed at a 40:1 ratio. The membrane was dried slightly and lied flat on a piece of cling film. The mixed solution was pipetted onto the membrane and incubated for 5 minutes. After either ECL detection system, the membrane was then 'blotted' dry,

placed in a plastic pocket in a light tight casset and exposed to Kodak film. The film was developed using an X-OMAT processor.

2.9.3 Quantification of signals

In order to determine the intensity of the signal the developed film was scanned into a computer, taking care to use an exposure which is in the linear range of the signal. Intensity of the bands were measured using the Molecular Analyst software (BioRad). For this boxes of equal size were drawn around each band of interest and the intensity of the area inside the box was measured. A background reading was taken and subtracted from the obtained value. Unless otherwise stated the mean and standard error of at least three such quantifications were calculated.

2.10 Endosome Ablation

2.10.1 Preparation of Tf-HRP Conjugate

Preparation of Tf-HRP protein conjugate was carried out as described by Livingstone *et al.* 1996.

Buffer 1 (0.1M NaCl/10mM $NaPO_4$, pH 7.2) was prepared by adding acid ($NaCl/NaH_2PO_4$) to alkali ($NaCl/Na_2HPO_4$) to pH 7.2. 150mg of horseradish peroxidase (HRP) was dissolved in 7.5ml of buffer 1, transferred to dialysis tubing (previously boiled in 2%$NaHCO_3$, 10mM EDTA and stored in 50% ethanol:50% H_2O) and dialysed at 4°C overnight against 2-4 litres of the same buffer. Following

dialysis, 3g of succinate (disodium salt) was added to the protein solution at room temperature. At 0°C, 1.05g of crushed succinic anhydride was added and the mixture was stirred for 30 min at 0°C and then for a further 30 min at room temperature.

Meanwhile, four 20ml G-50 sephadex columns were prepared. Buffer 2 (0.1M NaCl/1mM $NaPO_4$) was prepared by adding acid ($NaCl/NaH_2PO_4$) to alkali ($NaCl/Na_2HPO_4$) to pH 7.2. 9g of G-50 sephadex was slowly added to 100ml of buffer 2 and four 20ml syringes, plugged with glass wool, were packed with this sephadex and equilibrated with the same buffer.

The mixture was then resolved using 3 of the columns (the remaining column being stored at 4°C to be used later) and eluted with buffer 2. The elutant was then concentrated using Centriprep 3 columns (Amicon Ltd, UK) by centrifugation at 2000 x g, at 4°C to a final volume of 1.5-3ml. At 0°C, 375mg of N-hydroxy succinimide and 600mg of N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were added and the mixture was stirred at 0°C for 3 h. The resultant solution was then resolved using the remaining G-50 sephadex column as before.

At this stage the 'active' HRP was ready to be coupled to transferrin. This was carried out immediately to prevent hydrolysis of the active NHS esters. 6ml of 25mg/ml apo-transferrin (in buffer 2) and 10^6-10^7 cpm ^{125}I-transferrin were added to the elutant and stirred at 2°C for 48 h. The solution was then quenched with glycine and either stored at 2°C with the addition of azide or snap frozen and stored at -80°C.

Meanwhile, a Sephacryl S-300 column (1m in length) was equilibrated overnight with buffer 2 at 10ml/h. The conjugate was concentrated to 2-3ml as before and applied to the column. When the brown band of conjugate reached approximately half way down the column, 0.5ml fractions were collected. The fractions were then counted using a γ-counter and a graph of cpm versus fraction number was plotted. The fractions representing the first two peaks were pooled (the first two being the smallest of three peaks, with the third peak corresponding to unconjugated ^{125}I-transferrin) and concentrated to 3-4ml as before. The conjugate was reapplied to the column and fractions were collected and counted as before. On plotting a graph, the fractions representing the largest peak were pooled and concentrated to 3-4ml as before. The protein concentration was determined by a BCA assay (see Section 2.5) before the conjugate was iron loaded by adding 375µl of a 50mM $FeSO_4$ stock and 112.5µl of a 250mM $KHCO_3$ stock. The conjugate was then filtered through Whatman No.1 paper, aliquoted, snap frozen and stored at -80°C.

2.10.2 Use of Tf-HRP

Ablation of the recycling endosomal system was carried out as described by Livingstone *et al.* 1996.

During a 2 h incubation in serum-free DMEM, 3T3-L1 adipocytes were incubated with Tf-HRP conjugate (20µg/ml final concentration) for 60 min at 37°C. The cells were then placed on ice, washed gently three times over a period of 10 min with ice-cold isotonic citrate buffer (150mM NaCl, 20mM Tri-sodium citrate, pH 5.0) and once with ice-cold PBS (see Section 2.2.1). Chilling the cells prevented any further vesicle trafficking during the DAB cytochemistry while acid washing with citrate

buffer removed any cell-surface-attached Tf-HRP. 3,3'-diaminobenzidine (DAB) (2mg/ml stock prepared freshly in PBS, vortexed thoroughly and filtered through a 0.22 μm-pore-size-filter) was added at 100 μg/ml to all cells and H_2O_2 (final concentration 0.02% v/v) was added to experimental plates only. After a 60 min incubation at 4°C in the dark the reaction was stopped by washing the cells with ice-cold PBS.

2.11 Fractionation of cells

2.11.1 Subcellular Fractionation of 3T3-L1 Adipocytes

Subcellular fractions (PM, HDM, LDM and SP) were obtained as described for the fractionation of rat adipocytes by Piper *et al.* 1991, modified by Martin *et al.* 1994

Cells cultured on 10cm plates were either treated as stated below or incubated at 37°C in serum-free DMEM for 2 h. Following the transfer of plates onto ice the cells were washed 3 times with ice-cold HES buffer (see Section 2.2.3). On addition of 2ml HES buffer containing protease inhibitors (protease inhibitor cocktail tablet from Boehringer Mannheim) the cells were scraped and then homogenised with 20 hand strokes in a Dounce homogeniser. Homogenates were transferred to pre-chilled Oakridge Beckman centrifuge tubes and centrifuged at 19,000 x g for 20 min at 4°C.

The resulting pellet was resuspended in 1ml ice-cold HES buffer and layered onto 1ml of 1.12M sucrose in HES buffer in a Beckman tube and centrifuged at 100,000 x g for 60 min at 4°C in a swing out rotor. The upper layer was carefully taken off to

leave a brown band at the interface (plasma membrane) and a brown pellet (mitochondria/nuclei fraction). The plasma membrane fraction was transferred to an Oakridge centrifuge tube, resuspended in 15ml HES buffer and pelleted at 40,000 x g for 20 min at 4°C.

Meanwhile, the supernatant from the initial spin was centrifuged at 41,000 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 designated the low density microsomal fraction (LDM). The supernatant of this spin contained the soluble proteins. All membrane fractions were resuspended in HES buffer and together with the soluble proteins snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

2.11.2 Total Membrane Preparation

Cells cultured on 10cm plates were either experimentally treated or incubated at 37°C in serum-free DMEM for 2 h. Following the transfer of plates onto ice the cells were washed 3 times with ice-cold HES buffer (see Section 2.2.3). On addition of 2ml HES buffer containing protease inhibitors (protease inhibitor cocktail tablet from Boehringer Mannheim) the cells were scraped and then homogenised with 20 hand strokes in a Dounce homogeniser.

The homogenate was centrifuged at 180,000 x g for 60 min at 4°C. The pellet from this spin was dissolved in HES Buffer (200µl per plate starting material) and re-homogenised in a small Dounce homogeniser with 10 hand strokes in order to limit protein aggregation. The supernatant of this spin containing the soluble proteins was

also kept. The fractions were snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

2.12 Immunoadsorption of GLUT4 Vesicles

3T3-L1 adipocytes were fractionated as above with minor modifications. For preparation of intracellular (LDM) membranes for immunoadsorption, cells cultured on 10cm plates were incubated at 37°C in serum-free DMEM for 2 h at 37°C. The plates were transferred onto ice and washed 3 times with ice-cold HES buffer (see Section 2.2.3). On addition of 1ml of the same buffer containing protease inhibitors, the cells were scraped and then homogenised in a Dounce homogeniser as before. Homogenates were transferred to pre-chilled Oakridge centrifuge tubes and centrifuged at 41,000 x g for 20 min at 4°C to remove plasma membranes, mitochondria, nuclei and high-density microsomes. The resulting supernatant was centrifuged at 180,000 x g for 60 min at 4°C to yield a pellet containing the low density microsomal (LDM) membranes, which was resuspended in HES buffer (200µl HES per plate of cells) containing NaCl (100mM final). The resuspended pellet was homogenised in a small Dounce homogeniser with about 10 hand strokes and represents the starting material.

Protein A-Sepharose was made up by weighing out 100mg of solid into an eppendorf tube and adding HES buffer in order to rehydrate the Sepharose. The beads were then spun down at full speed for about 30 seconds and the supernatant removed, the beads were washed three times in HES buffer containing 1% bovine serum albumin (BSA). In order to make a 50% slurry HES containing 1%BSA was added to the swollen

protein A Sepharose. The beads were now blocked by adding about 500µl of HES containing 2% BSA to the beads and rotating them for 2hours at 4°C. After this time the blocked beads were spun down the supernatant was removed and the beads were then loaded with either affinity purified polyclonal anti-GLUT4 (see Section 2.4.1) or non-specific IgG, as a control, by shaking for 2 h on a miramax at 4°C.

In order to preclear the LDM's, 400µl of sample was added to an eppendorf tube together with 100µl of unloaded beads (washed once in HES) as well as 300µl HES containing 100mM NaCl. This was rotated on a wheel at 4°C for the same time as the beads were precoupled.

After this time the coupled beads were washed 3 times with HES containing 1% BSA. The tubes containing the LDM samples were spun down, the supernatant containing precleared LDM were added onto the coupled beads and the pellet was discarded. More HES/NaCl was added to the tubes and these were rotated slowly end-over-end for 3hours at 4°C.

After this time samples were centrifuged for 5 min at 2000 x g to pellet the immunadsorbent. The supernatant from this spin was centrifuged at 2000 x g for 10 min to remove any remaining cells. The resulting supernatant was added to 5ml Beckman Ultra-clear tubes and centrifuged at 180,000 x g for 60 min at 4°C. The resulting pellet containing LDM after adsorption was resuspended in 100µl HES (see Section 2.6).

The immunoadsorbent pellet was washed three times in 1ml HES/NaCl. The pellet was then resuspended in 90µl of 1% Triton X-100 in PBS, vortexed briefly and the

vesicle proteins were solubilised by incubation with occasional agitation for 30 min at room temperature. The beads were pelleted by centrifugation at 10,000 x g for 30 s using a microfuge. The supernatant containing the adsorbed vesicles was collected to a fresh tube and again centrifuged for 30 s before carefully removing the supernatant.

All fractions were snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

2.13 Indirect Immunofluorescence Microscopy

2.13.1 Plasma Membrane Lawn Assay

The plasma membrane lawn assay was carried out as described (Robinson *et al* 1992) to generate highly purified plasma membrane (PM) fragments in order to determine the relative levels of different proteins at the cell surface.

3T3-L1 adipocytes cultured on collagen-coated coverslips on 6-well plates were treated as described in individual experiments. At the conclusion of the experiment, cells were rapidly washed once with ice-cold buffer A (50mM HEPES (pH7.2) and 10mM NaCl) and then twice with ice-cold buffer B (20mM HEPES (pH7.2), 100mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, pepstatin A (1µg/ml), Trypsin inhibitor (2µg/ml) and 0.5mM Benzamide). While immersed in buffer B, the cells were sonicated using a 0.8mm tapered Dawa Ultrasonics probe at 50 watts for 3 s to generate a lawn of PM fragments attached to the coverslip.

Lawns were washed three times with PBS (see Section 2.2.1) and then fixed to the coverslips for 15 min by incubating in freshly prepared 2% paraformaldehyde in PBS. Cells were then washed three times with PBS and the reaction was quenched with 3 washes of 50mM NH_4Cl/PBS over a period of 10 min. Cells were washed a further three times with PBS. Cells were then washed with PBS/0.2% gelatin (PBS, 0.2% fish-skin gelatin and 0.1% goat serum, to block non-specific binding sites) and again three times with PBS over a 5 min period.

An aliquot of primary antibody was centifuged in a micofuge, diluted to the required concentration in PBS/0.2% gelatin and stored on ice prior to use. Following the addition of 100µl of antibody solution to a sheet of parafilm, the coverslips were placed cells-down on the solution and incubated at room temperature for 1-2 h. PM lawns were washed three times sequentially with PBS/0.2% gelatin and PBS over a 5 min period.

Due to fluorescent dyes on the secondary antibodies the preparation of PM lawns from this point on was continued in as dark a room as possible. Secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was prepared as for primary antibody and staining was carried out as described above. Coverslips were covered with aluminium foil during the 1-2 h incubation to avoid bleaching of dyes. As before, the PM lawns were washed three times sequentially with PBS/0.2% gelatin and PBS over a 5 min period. Coverslips were then mounted onto slides by placing them lawn down onto a 15µl drop of moviol. Again, to prevent bleaching the slides were covered with aluminium foil and allowed to dry overnight. The slides were stored in the dark prior to analysis.

Coverslips were viewed using x 40 or x 63/1.4 Zeiss oil immersion objectives on a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Germany) equipped with a Zeiss LSM4 laser confocal imaging system. For each coverslip about 8 different lawn areas were scanned into the computer and the intensity of the signal was determined using Metamorph software (Universal Imaging, Inc.,West Chester, PA) . Means and standard error of those values were calculated.

2.13.2 Whole cell Indirect Immunofluorescence Microscopy

3T3-L1 adipocytes cultured on coverslips on 6-well plates were either treated as stated below or incubated in serum-free DMEM for 2 h at 37°C. Cells were then transferred onto ice and washed three times in cold PBS (see Section 2.2.1) and then fixed using ice cold acetone for 15 min. Subsequently the cells were again washed three times with PBS, followed by three washes in PBS/5% goat serum over a time period of 30 min. The cells were then incubated with primary antibody diluted in PBS/1% goat serum for 1 hour as described above. After this time cells were washed three times with PBS/0.1% (w/v) BSA and incubated with FITC-conjugated secondary antibodies diluted in PBS/2.5% goat serum as described above. Cells were again washed three times with PBS and mounted on glass slides as described above.

2.14 Chronic Insulin treatment

Treatment with insulin for a prolonged time was as described by Kozka *et.al* 1991.

3T3-L1 adipocytes were cultured either on 6-well plates or 10cm dishes. Cells were then incubated with or without 500nM insulin for 24 h. After this time the plates were transferred onto a 37°C hotplate and washed 4 times over the course of about 1 h 20 min with KRMES Buffer pH6 (see section 2.2.4) (warmed up to 37°C) in order to remove all insulin from the receptors. After this time cells were quickly washed with warm PBS, serum free media was added and cells were transferred back into an incubator at 37°C (humidified atmosphere of 10% CO_2) for 2 h. The plates were then again placed on a 37°C hot plate, washed 3 times over half an hour with KRHEPES buffer (see section 2.2.5) and then stimulated in this buffer with or without 1μM insulin for 30 minutes. After this time subcellular fractionations, total membrane preparation, plasma membrane lawn assays or whole cell immunofluorescence were done as described above.

2.15 Iodixanol Gradients

OptiPrep is a sterile solution of Iodixanol (5,5'[(2-hydroxy-1-3propanediyl)-bis(acetylamino)]bis[N,N'-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarbox-amide].

Gradients were prepared as described by Hashiramoto *et al* 2000 with slight changes.

Low density microsomal (LDM) membranes were prepared as described before and mixed with iodixanol to a final concentration of 14%. Volumes were made up to 3.9ml using HES Buffer (see Section 2.2.3) and the mixture was sealed in quick-seal Beckman polyallomer tubes. Tubes were inverted several times to ensure sufficient mixing and then spun at 295 000 x g in a near vertical rotor (Beckman, TLN100) for 1 h at 4°C. Acceleration from 0 – 5000 rpm was set to be 5 min and decceleration was without breaks in order not to disturb the forming gradient. 300µl fractions were collected by piercing first a hole in the top of the tube to let air in and then piercing the bottom of the tube.

All fractions were snap frozen in liquid nitrogen and stored at -80°C prior to analysis.

2.16 Immunoprecipitations

Cells were grown on 10cm plates and treated as described in the results chapters. After the experiment, plates were transferred onto ice, washed once with cold PBS (see Section 2.2.1) and then 2ml of lysis/Immunoprecipitation buffer (see section 2.2.6) were pipetted onto the plate. The buffer was left on the plates for about 5 min and then cells were washed off the plates by pipetting the buffer up and down. The cell suspension was tranferred to chilled Beckman tubes and spun at 50 000 x g for 1 h at 4°C.

The pellet from this spin was discarded and the supernatant was used for immunoprecipitation. This supernatant was transferred to eppendorf tubes and incubated with the respective antibody (usually about 10µg of antibody per immunoprecipitation) by rotating on a rotating wheel at 4°C for 2 to 4 h. Protein A-Sepharose or protein G-Sepharose was made up by weighing out 100mg of solid into an eppendorf tube and adding Immunoprecipitation (IP) buffer in order to rehydrate the Sepharose. The beads were then spun down at full speed for 30 seconds and the supernatant removed. Now the beads were washed three times in IP buffer. In order to make a 50% slurry the same amount of IP buffer was added to the beads as there was pure beads. About 50µl of 50% slurry was added to each immunoprecipitation and the tubes were rotated for at least another 2 h at 4°C. After this time the immuoprecipitations were spun at 5000 x g for 2 minutes at 4°C and the supernatant was transferred to a fresh tube and snap frozen. The sepharose beads were washed once in IP buffer and once in IP buffer containing only 0.1% Triton. After removing all the liquid of the beads 100µl of 1.3 x sample buffer (120.9mM Tris-Cl pH 6.8,

1.3mM sodium EDTA, 13% (w/v) glycerol, 2.6% (w/v) SDS, 0.0026% (w/v) bromophenol blue, 6M Urea, 26mM dithiothreitol) were added to the beads and incubated at room temperature for 30 min. After this time the beads were spun down and discarded and the supernatant containing any immunoprecipitated proteins was snap frozen and stored at -80°C prior to analysis.

2.17 Yeast two hybrid

2.17.1 Transformation

Saccharomyces cerevisiae strain L40 (MATa his3D200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ gal4 gal80) was co-transformed with plasmids to express either the C- or N tail of GLUT4 as LexA fusions (pBTM116) and μ1, μ2, μ3A, μ3B or μ4 as VP16 fusions. As a negative control cells were also transformed with pLexA-GLUT4 N- or C terminus and pVP16.

For this 10ml of YEPD were inoculated with several colonies of L40 and grown over night at 30°C. 5mls of this overnight culture were then added to 50 mls of YEPD containing 2% glucose and grown for a further 2-4 h.The cells were then pelleted by spinning at 3500 x g for 5 min. The cells were washed once in TE (10mM Tris p H 7.5, 1mM EDTA) and pelleted as before. The cells were now resuspended in 2ml of a 100mM Lithium acetate/0.5 x TE mix (200μl of 1M LiAc pH 7.5, 100μl 10 x TE, 1.7ml dH_2O) and incubated in this for 10 min at room temperature. Meanwhile a DNA mixture was made in 1.5ml eppendorf tubes; for this 1μg of each plasmid

DNA (GLUT4 constructs and μ constructs) was mixed with 100μg of denatured salmon sperm DNA (denatured by boiling for 5 min immediately before use). After the 10 min incubation 100μl of yeast suspension was added to the DNA and mixed well. To this 700μl of a 100mMLiAc/40% PEG-3350/1 x TE mix (1.4ml 1M LiAc, 1.4ml 10 x TE, 11.2ml 50% PEG-3350) was added and mixed by vortexing.. This was now incubated for 30 min at 30°C. After this time 88μl of DMSO were added, mixed and incubated for 7 min at 42°C. The tubes were then microfuged at full speed for 1 minute, the supernatant was aspirated and the pellet was resuspended in 1ml TE. The tubes were again microfuged at full speed for 10 s and the supernatant aspirated. The cell pellet was now resuspended in 100μl of TE and plated on selective media.

In order to make Yc plates 1.2g yeast nitrogen base (lacking amino acids) 5g ammonium sulfate and 10g succinic acid were mixed in 900ml of water and pH'd to 5, then 2g of agar were added to each 90 ml of media and this was autoclaved. When the mixture had cooled down to slightly hotter then handwarm, glucose to a final concentration of 2% and amino acids to 0.0005% or 0.001% were added and plates were poured. For the selection, plates lacked uracil and the amino acids lysine in order to maintain the L40 strain, the amino acid tryptophan in order to select for the LexA-GLUT4 fusions and leucine in order to select for the VP16-μ fusions. Plates were grown for 3 days at 30°C.

2.17.2 β-Galactosidase Filter Assay

Overnight cultures of the transformations were grown in Yc media containing 2% glucose and all amino acids except Trp, Leu, Lys and uracil. One loop of these O/N cultures were streaked onto selective media lacking Trp, Leu, Lys and Ura and grown until visible colonies had formed. The colonies were then lifted onto whatman 1 filter paper by slowly lowereing the filter onto the plate and letting the yeast adsorb into the paper. The filter was then lifted off snap frozen in liquid nitrogen and then thawed at room temperature. This freeze/thaw cycle was repeated three times in order to break the cells open. 5-10ml of Z Buffer (60mM Na2HPO4, 40mM NaH2PO4, 10mM KCl, 1mM MgSO4, pH 7) containing 0.4mg/ml X-gal and 0.28% Mercaptoethanol were added to a new square plate and two fresh Whatman 1 filters were lowered onto the plate avoiding air-bubbles. Excess buffer was then drained and the thawed filter containing the yeast was laid on top of the two filter again avoiding air-bubbles. This was now incubated at 30°C overnight.

The development of blue colour was an indication of beta-galactosidase activity and therefore an interaction of the two fusion proteins.

2.17.3 -His plates

Overnight cultures of the transformations were grown in Yc media containing 2% glucose and all amino acids except Trp, Leu, Lys and uracil. One loop of these overnight cultures were streaked onto selective media lacking Trp, Leu, Lys, Ura and His (in order to select for positive interactants) and grown until colonies on the

positive controls appeared. Growth on media lacking histidine suggests an interaction of the fusion proteins.

2.17.4 -His liquid cultures

Overnight cultures of the transformations were grown in Yc media containing 2% glucose and all amino acids except Trp, Leu, Lys and uracil. Liquid -His cultures were then set up using 5 ml of YC media containing 2% glucose and all amino acids except Trp, Leu, Lys, His and uracil and adding 5µl of the overnight cultures. These cultures were grown in the shaking incubator at 30°C for up to 72 h. OD_{600} readings were taken at different time points in order to determine growth of the cultures. Growth in media lacking histidine suggests an interaction of the fusion proteins.

2.17.5 Liquid β-Galactosidase Assay

Overnight cultures of the transformations were grown in Yc media containing 2% glucose and all amino acids except Trp, Leu, Lys and uracil. 1ml of this overnight cuture was then added to 4ml of YPD media and grown until the cells were mid log phase (OD_{600} 0.5-0.8). 1.5 ml of the culture were then transferred into eppendorf tubes, centrifuged at 16 000 x g for 30 s to pellet the cells and the supernatant was aspirated. The cells were then resuspended in 1ml of Buffer 1 (100mM HEPES, 150mM NaCl, 5mM aspartic acid, 10mg/ml BSA, 0.05% Tween, pH 7.3), centrifuged again and the supernatant taken off. The pellet was then resuspended in 300µl of Buffer 1 and 100µl were transferred to a new tube. Cells were then broken open by repeated freeze thaw cycles (frozen in liquid nitrogen and then thawed at 37°C for ca 1 min, repeated three times) then 0.7 ml of Buffer 2 (20ml Buffer1 with

27.1 mg of Chlorophenol red-β-D-galactopyranoside (CPRG)) were added to each sample and mixed by vortexing. The time when buffer 2 was added was recorded and used as starting time for the colour to develop. Samples were then incubated at 30°C for several hours checking the development of colour regulary. The reaction was stopped by adding 0.5 ml 3mM $ZnCl_2$ to the sample and the tubes were then centrifuged at 16 000 x g for 1 min in order to get rid of cell debris. The OD_{578} of 1 ml sample was then measured and the β-galactosidase units were calculated:

β-galactosidase units = $1000 \times OD_{578}/(t \times V \times OD_{600})$

where: t = elapsed time (in min) of incubation

V = 0.1 x concentration factor (concentration factor = 5)

$OD_{600} = A_{600}$ of 1 ml starting culture

CHAPTER 3 - IODIXANOL GRADIENT ANALYSIS OF INTRACELLULAR GLUT4 STORES AND THE FORMATION OF THE GLUT4 COMPARTMENT DURING DIFFERENTIATION

3.1 Aims

1. To further characterise the use of iodixanol equilibrium centrifugation for resolving GLUT4 compartments in 3T3-L1 adipocytes

2. To examine the formation of the insulin responsive compartment during differentiation of 3T3-L1 fibroblasts into adipocytes

3.2 Introduction

Insulin stimulates the uptake of glucose into fat and muscle cells. This effect is mainly due to the translocation of GLUT4 from intracellular membranes to the cell surface in the presence of insulin (see Chapter 1.5). In the absence of insulin GLUT4 is found both in endosomes (~40%) and in specialised GLUT4 storage vesicles (GSV) (~60%) (Martin *et al.* 1997; Livingstone *et al.* 1996). The GSVs are highly insulin sensitive and explain why GLUT4 exhibits a much larger extent of translocation to the plasma membrane (about 15-fold) than other endosomal proteins such as the transferrin receptor (TfR) and GLUT1 (about 2-fold) (Tanner & Lienhard 1987; Piper *et al.* 1991).

The nature and formation of the GLUT4 containing compartment has been the subject of a great deal of research (for Review see Rea & James 1997).

The differentiation of 3T3-L1 fibroblasts into adipocytes involves morphological changes as well as changes in gene expression. The cells begin to round up and accumulate lipid droplets (Blok *et al.* 1988; El-Jack *et al.* 1999) and proteins necessary for the insulin-stimulated uptake of glucose are synthesised (Frost & Lane 1988). These proteins include insulin signalling proteins such as the insulin receptor and insulin receptor substrates (IRS) (Kohanski et al. 1986; Frost & Lane 1988) as well as GLUT4. GLUT4 protein can first be detected around day 4 of differentiation and its levels increase dramatically thereafter and plateau around day 10 (Birnbaum 1989; Kaestner *et al.* 1989). Other proteins such as the TfR and GLUT1 are present in fibroblasts and adipocytes (El-Jack *et al.* 1999).

The aminopeptidase IRAP (also called vp165) is present in fibroblasts, as well as adipocytes. Its level increases 8 - 10-fold during differentiation and it has been found to co-localise up to 90% with GLUT4 in adipocytes (Chapter 1.6). IRAP is ablated using Tf-HRP to a similar amount as GLUT4 and is known to translocate to the plasma membrane in response to insulin in a similar manner as GLUT4 (Ross *et al.* 1996; Martin *et al.* 1997). The body of evidence now available strongly suggests that in adipocytes and cardiac myocytes, GLUT4 and IRAP exhibit almost complete co-localisation.

One of the main problems in studying GLUT4 has been the difficulty of segregating GSVs from recycling endosomes. Endosome ablation has been used to determine the amount of GLUT4 present in the endosomes or the GSV compartment (Livingstone

et al. 1996) but this approach does not resolve the compartments for purification purposes. A recent study has described a new approach to separate these two vesicle populations using iodixanol gradient centrifugation (Hashiramoto & James 2000). Iodixanol is an iodinated density gradient compound which can form self-generated gradients. It can be added directly to the biological sample and is an ideal medium for the resolution of, for example, membrane vesicles (Billington *et al.* 1998). In 3T3-L1 adipocytes iodixanol gradient centrifugation has been used to separate intracellular GLUT4-containing membranes into two peaks, one being enriched in endosomal/TGN markers (peak 2) and the other containing the GSVs (peak 1) (Hashiramoto & James 2000).

In these studies we have characterised iodixanol gradient centrifugation in 3T3-L1 adipocytes with respect to GLUT4 and other proteins that partly co-localise with GLUT4. We have confirmed here that this is a powerful means to resolve distinct intracellular GLUT4 pools.

We have also used endosome ablation to study the formation of the GSV compartment during differentiation using IRAP as a marker protein due to its presence throughout differentiation. This data suggests that the GLUT4 storage compartment only forms during differentiation and only once GLUT4 is expressed.

3.3 Results

3.3.1 Characterisation of the iodixanol gradients

In order to visualise the two pools of GLUT4 (endosomal and GSV) we have used the method of Hashiramoto and James using iodixanol equilibrium sedimentation analysis (Hashiramoto & James 2000).

Due to the use of a near vertical rotor in this study instead of the vertical rotor used by Hashiramoto we found it necessary to change this method slightly and use a higher g-force but a shorter spin-time (*Materials and Methods, section 2.15*). Using this approach intracellular GLUT4 vesicle fractions from 3T3-L1 adipocytes were segregated into two peaks as can be seen in Figure 3.1A. Peak 1 contains 40 ± 5% of the total GLUT4 present within the LDM fraction and peak 2 contains 45 ± 8% of the GLUT4 present within the LDM fraction (mean ± S.D. of three experiments). In agreement with Hashiramoto and James, we found that insulin induced a greater loss of GLUT4 from peak 1 (51 ± 3.5% reduction) then from peak 2 (Figure 3.1B) consistent with the suggestion that peak 1 contains the GLUT4 storage vesicles. Peak 2 in contrast is thought to contain the recycling endosomes, and in response to insulin, GLUT4 levels in this peak decrease by 23 ± 2.6%, consistent with the idea that the endosomes are less insulin responsive than GSVs.

We also show here that IRAP behaves in a very similar manner to GLUT4. In the absence of insulin IRAP is present in both peaks, but in the presence of insulin there is a greater reduction of IRAP from peak 1 (61 ± 6.3% reduction) then peak 2 (30 ± 4% reduction) (Figure 3.1A and 3.1B).

In order to further characterise the nature of these two compartments, we examined the distribution of the v-SNAREs VAMP2 and cellubrevin in iodixanol gradient fractions. (Figure 3.1C) VAMP2 was found to co-segregate with the GSV peak and cellubrevin co-segregated with the endosomal peak (Figure 3.1C). This is somewhat different to the data of Hashiramoto, who found both proteins equally present in peaks 1 and 2, but supports the idea that VAMP2 is the v-SNARE for the GSVs, while cellubrevin is the v-SNARE for the endosomes (Martin *et al.* 1996; Martin *et al.* 1998). The transferrin receptor is found in both peaks and again insulin causes a greater loss of this protein from peak 1 (Figure 3.1C).

In order to determine the robustness of the separation of the two peaks, we centrifuged some of the fractions through a second iodixanol gradient. For this either fractions 2 and 3 or fractions 9 and 10 were pooled, subjected to iodixanol gradient centrifugation, and recovered fractions probed for GLUT4 content. As can be seen in Figure 3.2A GLUT4 in fractions 2 and 3 ended as predicted in the denser peak following re-centrifugation. On the other hand, GLUT4 in fractions 9 and 10, which originated from peak 2, distributed throughout the whole of the second gradient, and formed again two peaks. This may relate to the suggestion that peak 2 consists of two peaks, peak 2a being enriched in sortilin (Morris *et al.* 1998; Gould unpublished; Hashiramoto & James 2000) and the lighter peak 2b containing the TfR, rab4 and rab11 (Hashiramoto & James 2000). We did not confirm this in this study. Alternatively, it may imply that the difference in vesicle size and density between the two pools is very small and that a longer spin-time would accumulate more recycling endosomes in the bottom fraction.

Previously it has been found that fraction 1 (bottom of the gradient) contained the majority of protein loaded onto the gradient. It was suggested that this fraction comprises cytoskeletal protein complexes (Hashiramoto & James 2000). We confirmed that most of the protein content of the gradient was present in the dense fraction 1 (Figure 3.2B). Analysis of protein content of the fractions using BCA (see section 2.5) gave a similar result with most protein in fraction 1 (data not shown). Other studies from this laboratory have confirmed that this fraction contains cytoskeletal proteins (L.Chamberlain, S.Kane and G.W.Gould unpublished).

3.3.2 Formation of the GSVs upon differentiation of 3T3-L1 fibroblasts

Endosome ablation is a technique that has been used extensively to distinguish between the endosomal and the GLUT4 storage compartment (Livingstone *et al.* 1996 and Introduction section 1.5.3). We wished to use this approach to examine the biogenesis of the GSVs during the differentiation of adipocytes. Because a previous study (Hashiramoto & James 2000) had shown that the concentration of DAB could be a crucial determinant of the effectiveness of the reaction, we performed an experiment in which we tested different lengths of incubation with Tf-HRP conjugate (1 and 3 hours) and different DAB concentrations (100μg/ml and 1mg/ml). Ablation of the TfR did not change significantly after longer exposure to the conjugate or after increased DAB concentrations (Figure 3.3). For this reason we used a 1 hour exposure to Tf-HRP and 100μg/ml DAB for further experiments. These conditions were the same as those used previously in all studies from this lab.

In order to determine at what time after differentiation the GLUT4 storage compartment forms, we performed endosome ablation using the Tf-HRP conjugate.

Due to the fact that expression of GLUT4 becomes evident around day 4 following the initiation of differentiation, we used IRAP as a marker for GSV formation prior to day 4. In Figure 3.4 it can be seen that IRAP is highly sensitive to endosome ablation at days 1 and 3, implying that the majority of IRAP is localised to TfR-positive endosomes at these times. At day 5 of differentiation on the other hand most of IRAP is resistant to endosome ablation, consistent with the protein being localised in a distinct set of intracellular vesicles, presumably GSVs.

Other people in the lab have confirmed this idea by separating the intracellular membranes from 3T3-L1 adipocytes at different stages of differentiation on iodixanol gradient (P.A.Baker, S.Goodfellow and G.W.Gould). For completion this Figure is included in this study (Figure 3.5) and shows that at day 1 and 4 most IRAP is found in the endosomal pool, while at day 8 most of it is found in the GSV peak suggesting again an absence of the GSVs before day 5.

3.4 Discussion

In these studies we have confirmed and extended the use of iodixanol gradient centrifugation as a powerful method to separate the endosomal and the GLUT4 storage compartments. In response to insulin GLUT4 and proteins that are known to co-localise with GLUT4, such as IRAP and VAMP2, translocate from peak 1 of the gradient to a much higher degree than from peak 2. This supports the argument that peak 1 contains the GSVs, while peak 2 contains the recycling endosomes and also probably some elements of the TGN (Hashiramoto & James 2000).

Our studies further suggest that the formation of the GSVs during differentiation happens at around day 4 - 5. Before this time IRAP is found in endosomes together with the transferrin receptor. This is supported by the fact that IRAP is ablated to a much greater extent on day 1 and 3 post differentiation than is the case at day 5 or day 8 - 12 (Martin *et al.* 1997). Detectable expression of GLUT4 is first observed at around day 4 -5, and we therefore propose that either the GLUT4 storage compartment only forms once GLUT4 is expressed, or that at a stage concomitant with expression of GLUT4, IRAP is able to enter pre-formed GSVs from which it was until then excluded.

Studies from other workers in the laboratory using iodixanol gradients support this data (Figure 3.5). IRAP and the TfR are found in the endosomal peak at day 1 and day 4 post differentiation, but at day 8 IRAP as well as GLUT4 are found both in the endosomal as well as in the GSV peak, suggesting either the absence of GSVs prior to day 4, or an inability of GLUT4 and IRAP to enter these vesicles before this time. These data are illustrated in Figure 3.5 and was performed by an undergraduate student (Paul A. Baker).

The absence of an insulin-sensitive GLUT4 storage compartment before day 4 is also supported by a study examining the levels of IRAP at the plasma membrane in fibroblasts and adipocytes (Ross *et al.* 1998). It was found that IRAP in fibroblasts increases 1.8-fold at the plasma membrane in response to insulin, much less than the 8-fold increase observed in differentiated adipocytes. TfR levels on the plasma membrane on the other hand stay nearly identical during differentiation. This shows that differentiation leads to enhanced intracellular sequestration of IRAP and suggests that the formation of the insulin responsive compartment occurs at some

time during differentiation (Ross *et al.* 1998). From our data we would conclude that this time-point during differentiation coincides with the expression of GLUT4, and that at this time the GSVs are formed and IRAP is sequestered into these vesicles.

It is important to note that one possible explanation of the data in Figure 3.4 is that this may be a consequence of the levels of expression of IRAP relative to TfR. As levels of IRAP increase substantially during differentiation but levels of TfR remain relatively constant, it is possible that the inability to observe significant ablation of IRAP after day 3 is a function of an increased ratio of IRAP:TfR. However, we do not believe this is the case. The cross-linking (ablating) efficiency of this system is a function of the enzyme HRP, and is thus catalytic and not stoichiometric. Also, we have shown that these conditions are capable of ablating all of the GLUT1 expressed in these cells (Livingstone *et al.* 1996); GLUT1 is expressed at a level in considerable excess compared to TfR. Hence, we believe that the data presented in Figure 4.3 is a function of altered localisation of IRAP and not altered stoichiometry of expression.

Studies using the transfection of GLUT4 into fibroblasts (Haney *et al.* 1991; Hudson *et al.* 1992) have shown that ectopically expressed GLUT4 does not translocate to the plasma membrane in response to insulin. Confocal staining and EM studies of those cells showed GLUT4 to be in structures which seemed identical to the GLUT4 compartments of adipocytes. Interestingly though the transfected GLUT4 co-localised totally with the TfR (Hudson *et al.* 1992). This suggests that the transfected GLUT4 is localised in endosomes, and the absence of GSVs in fibroblasts.

Other studies have proposed that the formation of the insulin responsive compartment occurs before the expression of GLUT4, at around day 3 of differentiation (El-Jack *et al.* 1999). Their conclusion is based on the narrowing of intracellular GLUT1 and TfR distributions in sucrose gradients from day 0 to day 3 of differentiation. IRAP is also found in the same peak on the gradient at day 3 and so is GLUT4 at day 9. Their conclusion is that this newly formed compartment is the GSVs, but the problem with sucrose gradient fractionations is that one cannot distinguish between endosomes and GSVs. Hence, in the study of El-Jack *et al.*, it is not possible to precisely determine where the GLUT4 is localised relative to TfR in any of the experiments. In 3T3-L1 adipocytes the amount of GLUT4 and TfR co-localisation is known to be about 30 - 40 % (Livingstone *et al.* 1996; Kandror & Pilch 1998).

Many studies now appear to support the notion that the majority of GLUT4 does not co-localise with the TfR in adipocytes or muscle, and evidence is accumulating to suggest that GLUT4, TfR and other plasma membrane proteins may recycle via multiple distinct pathways. The key issue is that the recycling of GLUT4 is largely distinct from these pathways and is profoundly insulin-sensitive (Tanner & Lienhard 1987; Holman *et al.* 1994; Kandror & Pilch 1998). This is discussed further below.

Malide & Cushman have used wortmannin in rat adipocytes in order to follow GLUT4 recycling in these cells. The PI3 kinase inhibitor wortmannin is known to block the translocation of GLUT4 to the plasma membrane in response to insulin (Chapter 1.3.3) and also causes the formation of phase-lucent vacuoles in rat adipocytes (Malide & Cushman 1997). In this study it was shown by immunofluorescence microscopy that when insulin was added together with

wortmannin GLUT4 stayed in vesicles identical to those in basal cells. On the other hand if insulin was given first followed by wortmannin several minutes later, GLUT4 accumulated in the wortmannin-induced vacuoles. The rational behind this was suggested to be that GLUT4 translocates to the plasma membrane and is then endocytosed, but due to the presence of wortmannin cannot be recycled further. The morphologically altered compartment is though to represent the endosomes, and GLUT4 was under these conditions found to co-localise with the TfR and rab4, both usually found in early endosomes. The conclusion of this study was that under normal conditions GLUT4 is sorted out of the TfR compartment into a separate vesicle population from which it can then translocate to the plasma membrane in response to insulin (Malide & Cushman 1997).

A recent study in rat adipocytes using cell surface biotinylation together with GLUT4 vesicle adsorption found that the TfR and IRAP present in GLUT4 vesicles behaved in very different ways in response to insulin. The TfR rapidly recycled between the plasma membrane and the GLUT4 vesicles and after only 5 minutes 50% of TfR was biotinylated corresponding with its appearance at the plasma membrane. In 30 minutes all the TfR found in the vesicles had been at the plasma membrane. IRAP on the other hand stayed on the plasma membrane for at least 30 minutes after insulin administration (Kandror 1999). This data suggests that, as shown before (Livingstone *et al.* 1996) GLUT4 is present in endosomes as well as GSVs, and that the difference in recycling rates between IRAP and TfR is due to their localisation in two different compartments.

These experiments all support the presence of GLUT4 in two different compartments, one being endosomes and the other a highly insulin sensitive

compartment, which we propose to be formed only once GLUT4 is expressed. Some of the data could also be interpreted that the GSVs are already present in fibroblasts, but only after the expression of GLUT4 do IRAP and GLUT4 get sequestered into this compartment. This seems rather unlikely though as we can find no role of this compartment in fibroblasts and also because there is clearly some kind of compartment formation at day 3 post differentiation (El-Jack *et al.* 1999).

It seems likely from these experiments that there are many steps which lead to the formation of the GSVs. During differentiation some kind of rearrangement of the cell occurs which leads to the formation of a different kind of endosomal recycling vesicle than present in fibroblasts. Expression of GLUT4 could then lead to the formation of the GSVs and sequestration of proteins such as IRAP away from the recycling endosomes. Clearly further work is required to elucidate this problem.

In conclusion we have confirmed the use of iodixanol gradient centrifugation as an important tool to study GLUT4 trafficking. We also present preliminary results to suggest that the formation of the GLUT4 storage compartment during differentiation happens only once GLUT4 is expressed.

Figure 3.1A Iodixanol gradient sedimentation analysis of GLUT4 and IRAP in 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated with (I) or without (B) 1μM insulin for 30 minutes. LDM fractions were prepared and subjected to iodixanol density gradient analysis as described in *Materials and Methods (section 2.15).* Fractions were collected from the bottom of the gradient and ca. 1/10 of each fraction was separated by SDS-PAGE. Shown are typical immunoblots for the proteins indicated in basal and insulin treated cells. Peak 1 corresponds to the GLUT4 storage vesicles (GSV) and peak 2 corresponds to endosomes/TGN.

Figure 3.1A

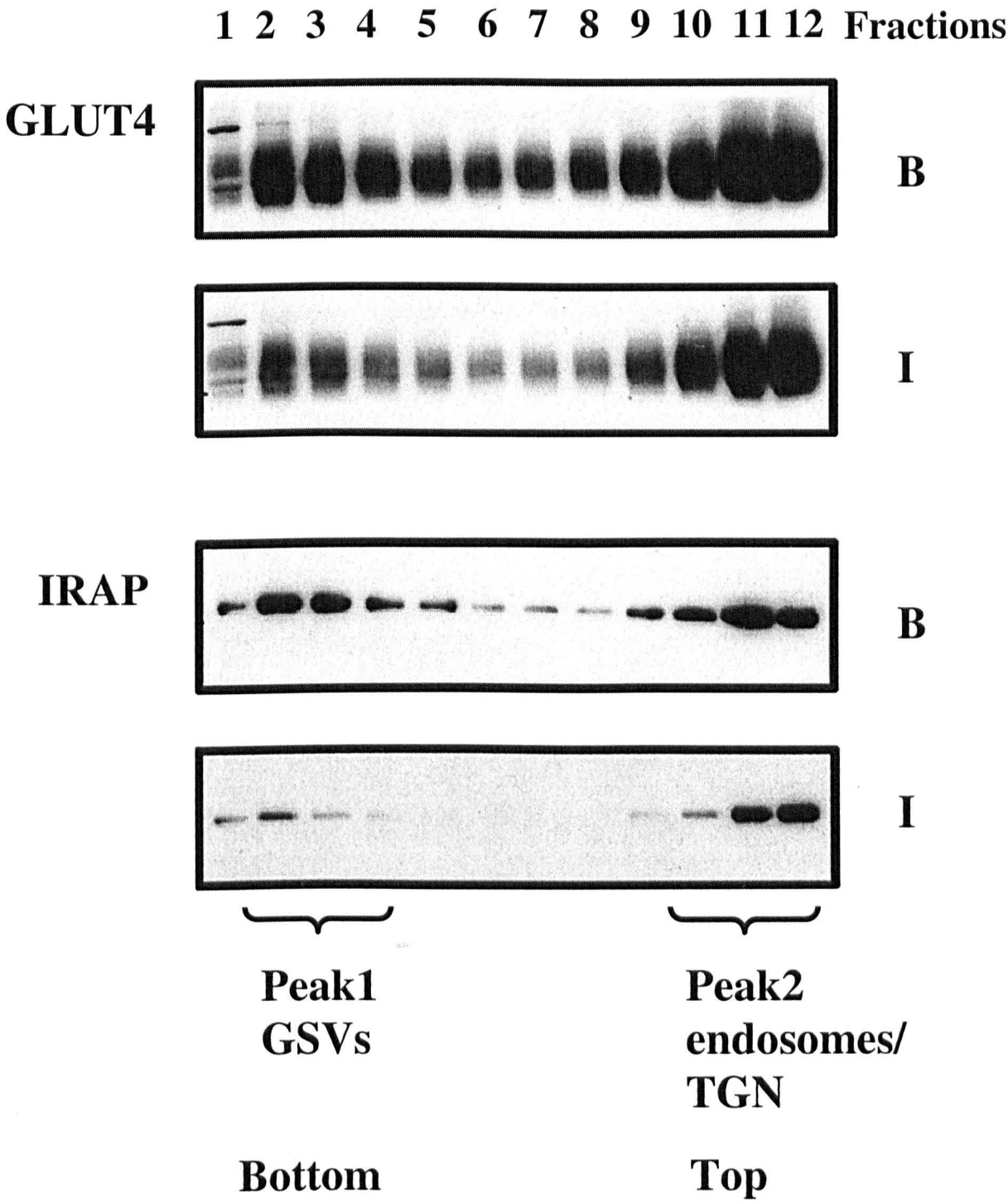

1 2 3 4 5 6 7 8 9 10 11 12 Fractions
GLUT4
B
I
IRAP
B
I
Peak1
GSVs
Peak2
endosomes/
TGN
Bottom
Top

Figure 3.1B Quantification of GLUT4 and IRAP on iodixanol gradients

3T3-L1 adipocytes were treated with (I) or without (B) 1μM insulin for 30 minutes. LDM fractions were prepared and subjected to iodixanol density gradient analysis as described in *Materials and Methods (section 2.15).* Samples were analysed as described in figure 1A and shown is the quantification of three independent experiments of this type in which the % change in GLUT4 and IRAP in each of the two peaks was quantified. The greater magnitude of reduction in peak 1 compared to peak 2 was significant for both proteins ($p<0.05$).

Figure 3.1B

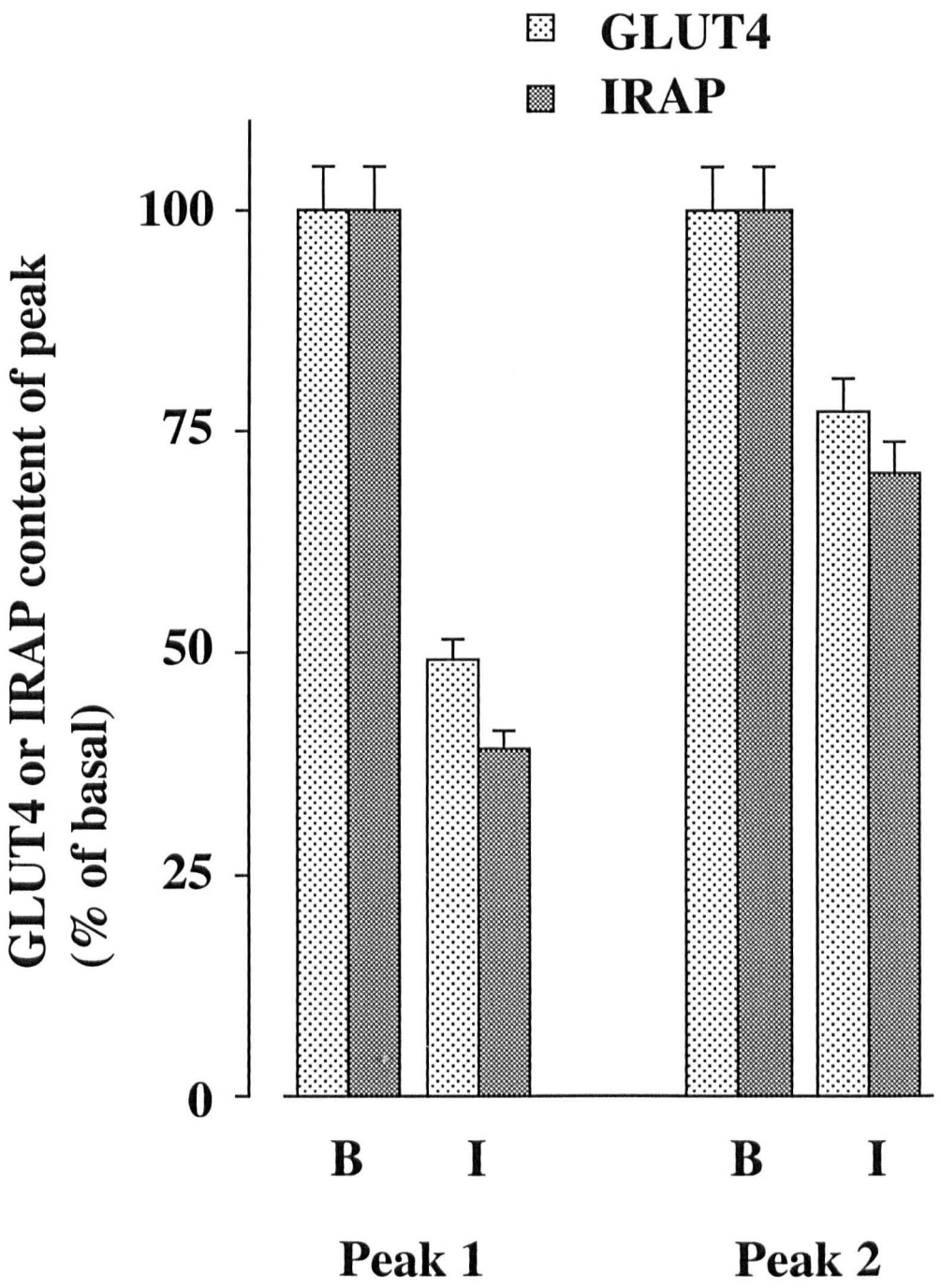

GLUT4
IRAP
GLUT4 or IRAP content of peak
(% of basal)
100
75
50
25
0
B
I
B
I
Peak 1
Peak 2

Figure 3.1C Iodixanol gradient sedimentation analysis of cellubrevin, VAMP2 and the transferrin receptor in 3T3-L1 adipocytes.

3T3-L1 adipocytes were treated with (I) or without (B) 1μM insulin for 30 minutes. LDM fractions were prepared and subjected to iodixanol density gradient analysis as described in *Materials and Methods (section 2.15).* Fractions were collected from the bottom of the gradient and ca. 1/10 of each fraction was separated by SDS-PAGE. Shown are typical immunoblots for the proteins indicated in basal and insulin treated cells.

Figure 3.1C

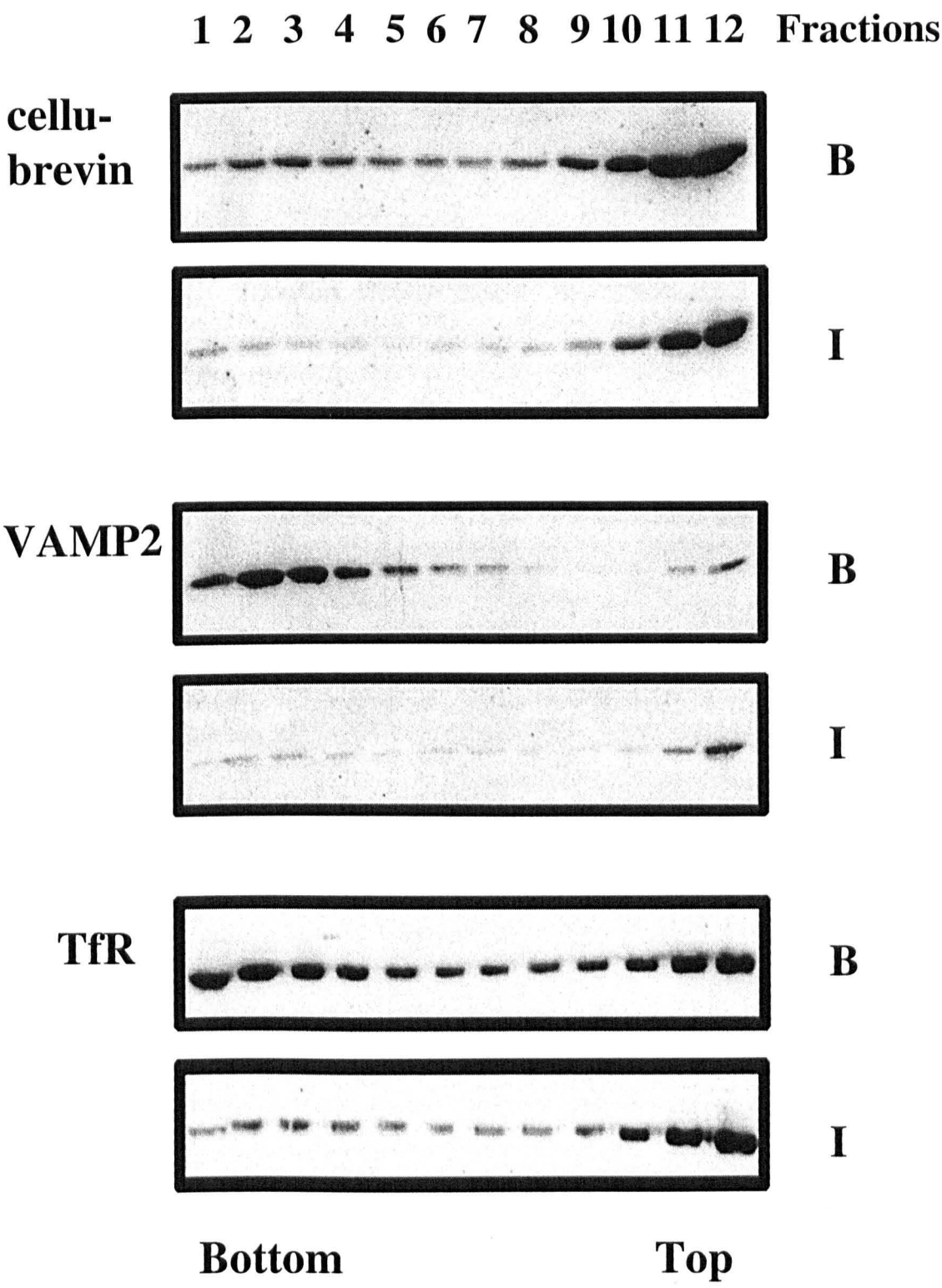

1 2 3 4 5 6 7 8 9 10 11 12 Fractions
cellu-
brevin
B
I
VAMP2
B
I
TfR
B
I
Bottom
Top

Figure 3.2A GLUT4 stays in the bottom but not top fractions when recentrifuged on a second iodixanol gradient

2 fractions of an iodixanol gradient (either fractions 2 and 3 or fractions 9 and 10) were pooled and recentrifuged on a second iodixanol gradient, as described in *Materials and Methods (section 2.15)*. The collected fractions were separated by SDS-PAGE and probed for GLUT4. Shown is a representative immunoblot (n=2).

Figure 3.2A

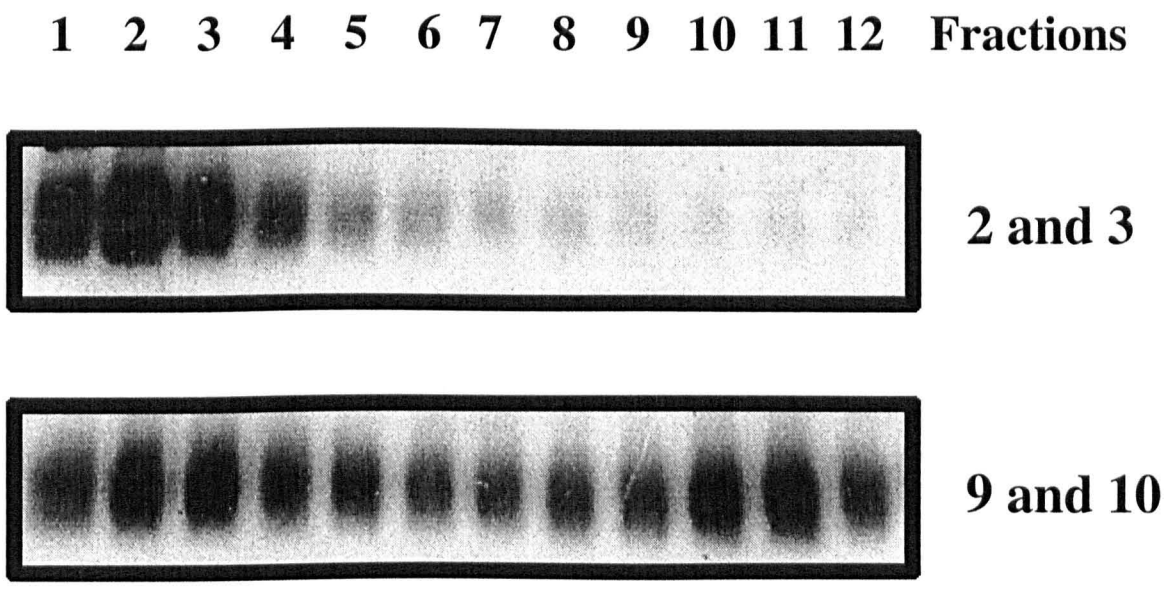


Figure 3.2B Protein profile of Iodixanol gradient fractions

Internal membranes (LDM's) from 3T3-L1 adipocytes were prepared and separated on an iodixanol gradient as described in *Materials and Methods (section 2.11.1 and 2.15).* The collected fractions were separated by SDS-PAGE and the gel was stained using coomassie blue. Shown is a representative gel. Protein estimation via BCA assay *(Materials and Methods section 2.5)* gave a similar result, with most protein in fraction 1.

Figure 3.2B

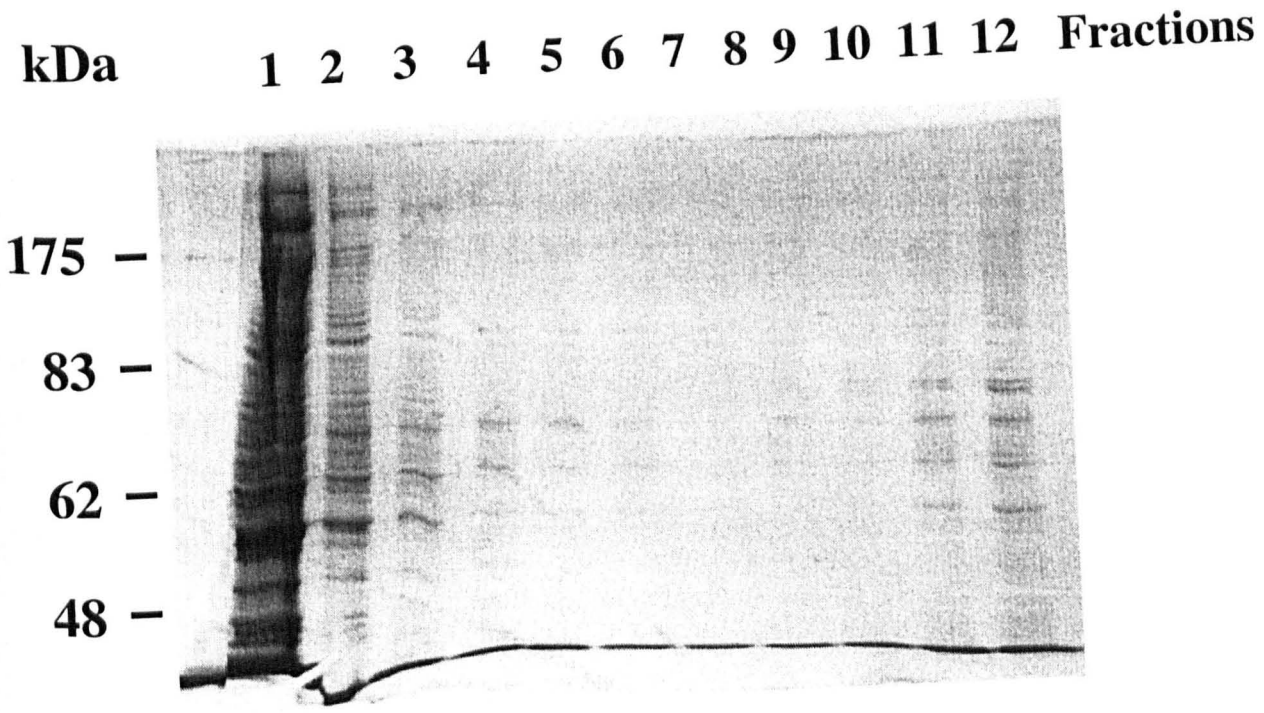


Figure 3.3 Prolonged incubation with Tf-HRP conjugate or higher DAB concentrations does not change the amount of endosome ablation in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated for either 1 hour (1h) or 3 hours (3h) with Tf-HRP conjugate and ablation was continued as described in *Materials and Methods (section 2.10.2)*, with the exception that DAB concentrations were varied. "+" indicates 1mg/ml DAB while for the other samples 100μg/ml DAB was used. Intracellular membranes (LDM's) were prepared from each sample and 20μg of protein was separated by SDS-PAGE, followed by immunoblotting with GLUT4 or TfR antibodies (TfR = transferrin receptor). Controls were incubated either with 100μg/ml (B) or 1mg/ml (B+) DAB. Shown is a typical experiment.

Figure 3.3

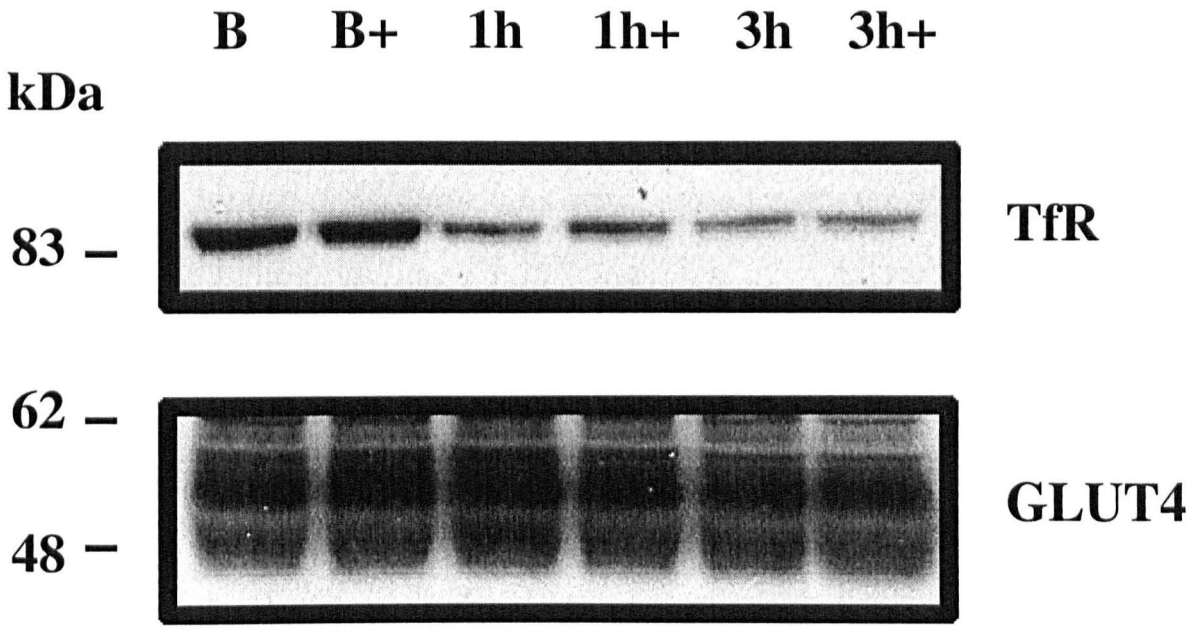


Figure 3.4 Ablation of IRAP, TfR and GLUT4 at different times after differentiation

Duplicate plates of 3T3-L1 adipocytes at 1, 3, and 5 days post-differentiation were loaded with Tf-HRP for 1 hour. Thereafter, cells were either ablated (+ H_2O_2) or not (-H_2O_2) as described in *Materials and Methods (section 2.10.2).* LDM's were prepared and for each condition 20μg of protein was separated by SDS-PAGE and immunoblotted incubated with the antibodies indicated. A similar result was obtained in a previous experiment using only cells at day 3 and day 5 after ablation.

Figure 3.4

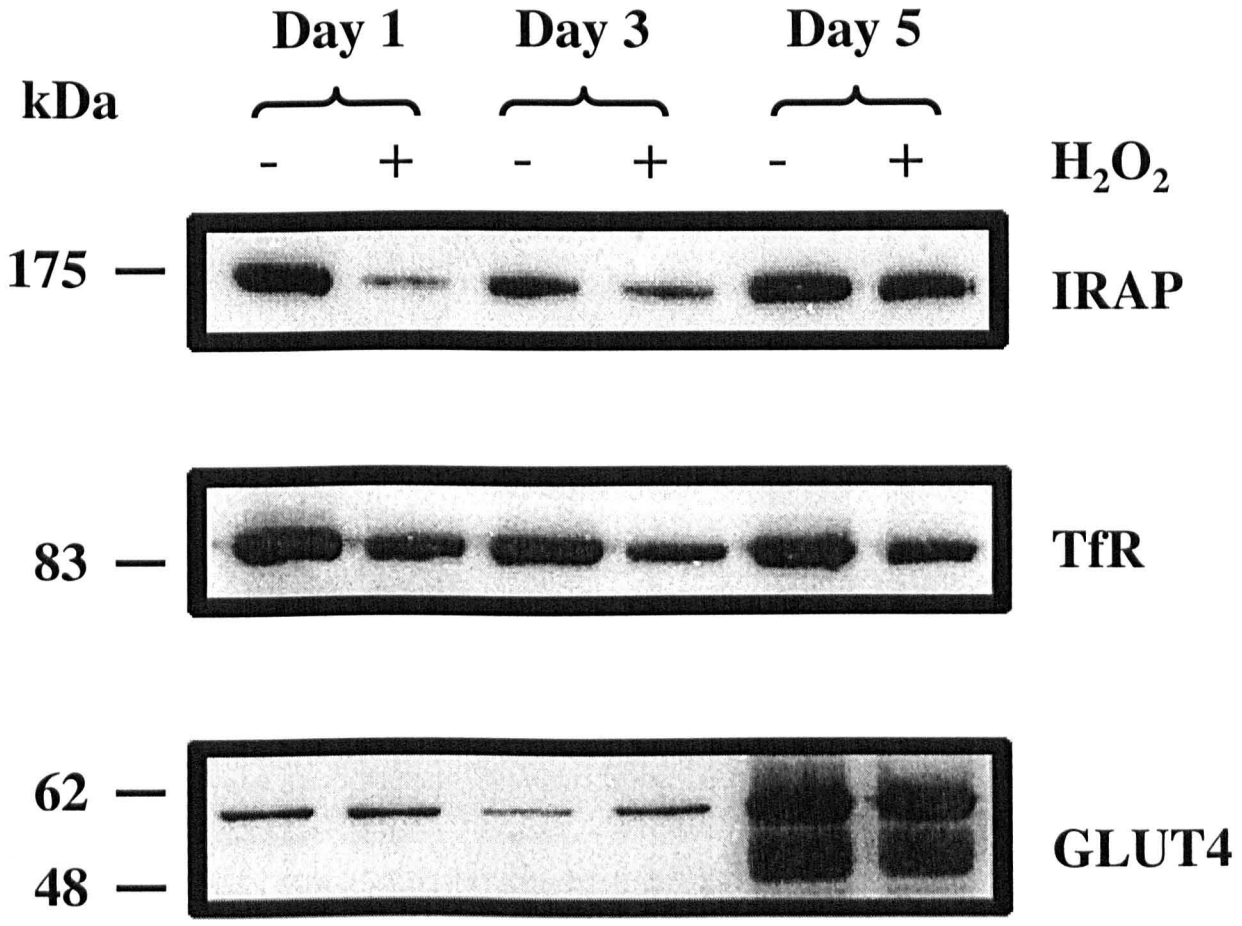
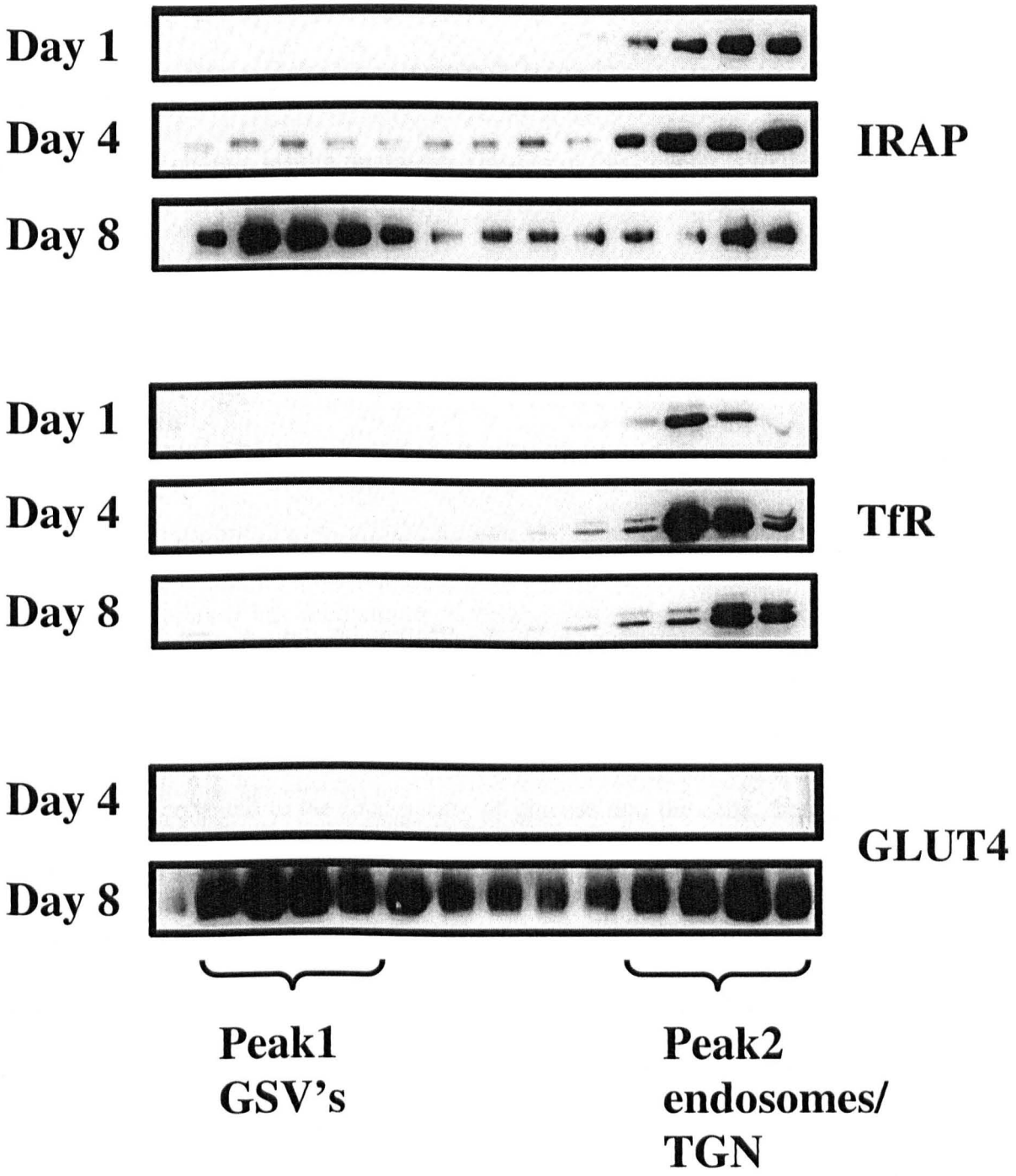


Figure 3.5 Iodixanol gradients of 3T3-L1 adipocytes at day 1, day 4 and day 8 after differentiation

Low density membranes of 3T3-L1 adipocytes were prepared as described in *Materials and Methods (section 2.11.1)* at either day 1, day 4 or day 8 after differentiation of the cells. Iodixanol gradients of the LDM's were run as described in *Materials and Methods (section 2.15)* and about 1/10th of the collected fractions were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (This Figure is from an experiment done by Paul A. Baker and Sarah Goodfellow)

Figure 3.5


Day 1
Day 4
Day 8
IRAP
Day 1
Day 4
Day 8
TfR
Day 4
Day 8
GLUT4
Peak1
GSV's
Peak2
endosomes/
TGN

CHAPTER 4 - GLUT4 TRAFFICKING IN THE INSULIN RESISTANT STATE

4.1 Aims

Insulin-stimulated GLUT4 translocation is known to be reduced in 3T3-L1 adipocytes after chronic insulin treatment. The aim of this chapter is to determine the molecular basis of this.

4.2 Introduction

Type 2 diabetes is one of the major health problems in the Western world (Chapter 1.2). In recent years it has been shown to be a very heterogeneous disease with no clear genotype leading to the phenotype of hyperinsulinaemia and hyperglycaemia. In the cascade of events, from insulin distribution in the blood to the effect it has on muscle and fat cells and to the final uptake of glucose into the cells, there are many possible sites of defect which could lead to an insulin resistant phenotype. The major downstream event which seems to be defective in all insulin resistant patients is the translocation of the insulin responsive glucose transporter GLUT4 from an intracellular store to the plasma membrane (as reviewed in Hunter & Garvey 1998). Many studies have therefore concentrated on understanding this translocation mechanism and on characterising the GLUT4 compartment.

In the absence of insulin 95% of GLUT4 is intracellular (Slot *et al.* 1991a; Slot *et al.* 1991b) and it is now known that it occupies at least two different intracellular compartments (Holman *et al.* 1994). One of these is thought to be the endosomes and the other is a highly insulin sensitive compartment sometimes named GLUT4 storage vesicles (GSVs) (Chapter 1.5.3). The presence of GLUT4 in these specialised vesicles enables the rapid translocation of GLUT4 to the plasma membrane in response to insulin and distinguishes this protein from endosomal proteins such as the transferrin receptor (TfR) and GLUT1 (Tanner & Lienhard 1987; Calderhead *et al.* 1990; Piper *et al.* 1991).

Some other proteins co-localise with GLUT4 in this specialised compartment, such as the insulin responsive aminopeptidase IRAP (sometimes called vp165). IRAP shows about a 8-fold translocation to the plasma membrane in response to insulin in 3T3-L1 adipocytes (Ross *et al.* 1996), and 90% of it has been shown to co-localise with GLUT4 within intracellular membranes of adipocytes and cardiac myocytes (Ross *et al.* 1997; Martin *et al.* 1998).

Another protein that co-localises with GLUT4 and IRAP in the GLUT4 storage vesicles is VAMP2. GLUT4 has been shown to translocate to the plasma membrane via a SNARE type mechanism (Cheatham *et al.* 1996; Rea *et al.* 1998) (Chapter 1.9). VAMP2 has been suggested to be the v-SNARE responsible for GLUT4 translocation from the GSVs, while cellubrevin is thought to be the v-SNARE involved in translocation of GLUT4 and other proteins from the endosomes to the plasma membrane (Martin *et al.* 1996; Martin *et al.* 1998; Miller *et al.* 1999). The corresponding t-SNAREs are syntaxin4 and SNAP23/syndet (Rea *et al.* 1998) and these are found mostly on the plasma membrane.

In this chapter we sought to determine why GLUT4 does not translocate to the plasma membrane in insulin resistant cells. Kozka *et al.* have shown that 3T3-L1 adipocytes treated for a prolonged time with insulin had 50% lower GLUT4 levels at the plasma membrane than control cells. These cells were also found to be resistant to acute insulin challenge and no further GLUT4 could be recruited to the plasma membrane (Kozka *et al.* 1991).

We confirm here that 3T3-L1 adipocytes can be made unresponsive to further insulin challenge when treated for a prolonged period of time with high levels of insulin. We further show that this chronic insulin treatment can cause changes in both the levels and subcellular distribution of many proteins involved in GLUT4 trafficking, and that GLUT4 becomes selectively depleted from the GSVs in insulin resistant cells.

4.3 Results

It has been shown that treatment of 3T3-L1 adipocytes with 500nM insulin for 24h followed by a reversal using low pH washes, renders these cells insulin resistant in terms of insulin-stimulated GLUT4 translocation (Kozka *et al.* 1991) (*Materials and Methods, section 2.14.1*). We first set out to recapitulate this data using the following basic protocol. Cells were incubated with 500nM insulin for 24h, and following this were washed with KRMES buffer (pH 6) for about 80 minutes to remove all insulin from its receptor. Cells were then incubated in serum free media for 2h and either treated or not with 1μM insulin in KRHEPES buffer (pH 7.4).

4.3.1 Effect of chronic insulin on GLUT4

To confirm previous published data regarding the effects of chronic insulin treatment on GLUT4 trafficking, we performed subcellular fractionations of control cells and chronically insulin treated cells and analysed these by SDS-PAGE and immunoblotting. It can be seen in Figure 4.1A that as expected in control cells GLUT4 translocates from the LDM fraction to the PM in response to an acute insulin treatment. On the other hand in cells which have been treated with insulin for 24h, the extent of GLUT4 translocation is greatly reduced and the levels of GLUT4 at the PM are less then those observed in acutely stimulated control cells.

When a subcellular fractionation of control and chronically insulin stimulated cells was separated by SDS-PAGE followed by silver staining, we could see that treatment of these cells with 500nM insulin for 24 hours did not significantly change the general protein composition of these cells (Figure 4.1B).

Similar results as mentioned above were obtained using the plasma membrane lawn assay of GLUT4 translocation (Figure 4.2A) which also showed a reduction in insulin-stimulated GLUT4 translocation in chronically insulin treated cells. The amount of GLUT4 at the plasma membrane was measured and it can be seen in the quantification in Figure 4.2B that while in control cells the fold increase at the PM due to acute insulin is about 6-fold, this is reduced to only 1.5-fold in chronically stimulated cells. Whole cell immunoflurescence gave very similar results (Figure 4.2C). Insulin clearly causes an increase of GLUT4 at the plasma membrane, but in chronic insulin treated cells there is less GLUT4 found at the plasma membrane and this was not significantly increased by an acute insulin challenge.

In order to determine whether the reduction in translocation of GLUT4 could be due to a decrease in total GLUT4 levels, total membranes from control and chronically treated cells were compared by quantitative immunoblotting. It can be seen from Figures 4.3A and B that GLUT4 levels are indeed down by about 50% in the chronic insulin treated cells.

Chronic insulin treatment therefore causes both a downregulation of GLUT4 protein and a decreased extent of insulin-stimulated translocation. This data is in agreement with published studies (Kozka *et al.* 1991; Kozka & Holman 1993; Sargeant *et al.* 1993; Flores-Riveros *et al.* 1993)

4.3.2 IRAP behaves differently to GLUT4 upon chronic insulin treatment

IRAP has been found to co-localise with GLUT4 and translocate in a similar manner as GLUT4 to the PM in response to insulin (Chapter 1.6). In contrast to GLUT4 however, cellular levels of IRAP were not depleted by chronic insulin treatment (Figure 4.3). Despite this, like GLUT4, IRAP exhibited decreased translocation from the LDM to the PM after chronic insulin treatment, as can be seen in Figure 4.1A. Furthermore, levels of IRAP at the PM in the absence of acute insulin are higher in chronic insulin cells compared to control cells (Figure 4.2B). Interestingly, IRAP levels in the HDM's also increased due to this treatment and this is in contrast to GLUT4 which is found to have a decrease in its levels in the HDM (Figure 4.1A).

4.3.3 Chronic insulin has an upregulatory effect on SNARE proteins

Although the decreased GLUT4 levels could explain the reduced levels of GLUT4 at the PM, they cannot explain the decreased fold translocation of GLUT4. This led us to propose that in insulin resistant 3T3-L1 adipocytes, GLUT4 is either mis-targeted and is not localised to the GSVs, or that there is a defect in the translocation of the GSVs to the plasma membrane.

The latter hypothesis led us to investigate proteins which have been implicated in GLUT4 trafficking. SNARE proteins are thought to be important for the docking and subsequent fusion of the GSVs with the plasma membrane (Introduction 1.9). We therefore examined different SNARE proteins both for their total cellular content and for their subcellular distribution.

The v-SNARE VAMP2, thought to be involved in the fusion of the GSV with the PM, is one such candidate. In Figure 4.3 it can be seen that VAMP2 levels are upregulated due to chronic insulin treatment (1.5-fold, $p < 0.02$). Analysis of subcellular fractionations showed that in chronic insulin treated cells VAMP2 levels at the PM do not change upon acute insulin exposure, and are higher than the corresponding fractions from control cells (Figure 4.4). Note that the fractionation experiment also demonstrates that VAMP2 levels are increased in all fractions in chronic insulin samples (Figure 4.4). By contrast, chronic insulin treatment did not result in a significant increase in cellubrevin content (Figure 4.3) or change its distribution (Figure 4.4).

Syntaxin4 the corresponding t-SNARE is found mostly at the plasma membrane and its levels due to chronic insulin are upregulated by 1.3-fold ($p < 0.05$) (Figure 4.3A and B). SNAP23/syndet another t-SNARE recently shown to be involved in GLUT4 trafficking (Rea *et al.*1998) was upregulated upon chronic insulin treatment by 50% ($p < 0.04$) (Figure 4.3A and B). Figure 4.3A and B also show, that levels of the transferrin receptor (TfR) and TGN38 do not change in response to chronic insulin treatment. The subcellular distribution of syntaxin4 and syndet were not greatly altered by chronic insulin.

4.3.4 Effect of chronic insulin on other proteins involved in GLUT4 trafficking

Targeting of cellular proteins to the right compartment is crucial for a cell and we therefore examined whether prolonged insulin treatment affected the distribution of proteins known to regulate membrane protein trafficking.

AP2, the adaptor known to be important for clathrin mediated endocytosis (as reviewed in Robinson 1994) is found mainly at the PM, as can be seen in Figure 4.5. Chronic insulin treatment does not change this distribution. AP1 has been found to localise to the TGN (as reviewed in Robinson 1994) and in Figure 4.5 it can be seen that this protein is found as expected in the LDM fraction. AP1 localisation does not seem to change due to chronic nor acute insulin.

4.3.5 Ablation analysis of endosomal GLUT4 content in control and chronic insulin treated cells

In order to test the hypothesis that GLUT4 is mis-targeted in insulin resistant cells, we investigated whether the distribution of GLUT4 between the GSVs and the endosomes changes in chronic insulin treated cells using endosome ablation (see Materials and Methods section 2.10.2).

Ablation of the endosomal recycling compartment using a Tf-HRP conjugate has been shown to eliminate most of the vesicles containing the transferrin receptor as well as endosomal markers such as cellubrevin and rab5 (Livingstone *et al.* 1996). Only about 40% of GLUT4 is ablated by this means, due to the fact that the majority of GLUT4 is localised in a specialised transferrin receptor negative compartment termed GSVs. We used ablation in order to determine whether under chronic insulin conditions more GLUT4 is localised in the endosomal recycling system than before, as this could account for decreased insulin-stimulated GLUT4 translocation, as the endosomes are inherently less insulin sensitive than GSVs (Millar *et al.* 1999). The results show no clear difference in the extent of ablation of GLUT4 in control or chronically insulin stimulated cells (Figure 4.6). When the same samples were probed for the transferrin receptor and IRAP, ablation of these proteins was shown to be increased in the chronically insulin stimulated cells, compared with control cells. Loading of cells with transferrin-HRP conjugate for one hour only ablates about 70% of the transferrin receptor (Livingstone *et al.* 1996). The increased ablation of the TfR after prolonged insulin treatment probably reflects the fact that there is an increase in endosomal recycling under these conditions and the conjugate is accessible to a larger fraction of the transferrin receptor.

These results would suggest little difference in the localisation of GLUT4 between chronically insulin stimulated cells and control cells, but that there is an increase of IRAP in the endosomes. This will be discussed further below.

4.3.6 Comparison of GLUT4 vesicles in control and chronic insulin stimulated cells

Another method to determine whether GLUT4 is mis-targeted in insulin resistant cells is to examine the composition of GLUT4 vesicles in control and chronic insulin treated cells. Such vesicles can be isolated by immunoadsorption approaches (see Materials and Methods section 2.12)

In Figure 4.7 the results of GLUT4 vesicle adsorption's are presented. In this method protein A-coated sepharose beads were loaded with GLUT4 antibody and then incubated with the LDM fraction of either control or chronically insulin treated cells. GLUT4 vesicles are separated from the LDM fraction by this means and it can be seen that nearly all the GLUT4 has disappeared from the LDM's after adsorption (lane 5 versus 6 and 7 versus 8), indicating that this method results in quantitative adsorption of GLUT4 from the LDM fractions. The use of a random IgG shows that there is no non-specific binding of GLUT4 vesicles to the sepharose beads.

The amount of IRAP found in GLUT4 vesicles did not change due to chronic insulin treatment (Figure 4.7A). Interestingly, more TfR seems to be present in the GLUT4 vesicles from chronic insulin treated cells. This would therefore suggest an increased co-localisation of GLUT4 and IRAP with the TfR.

We have silver-stained the GLUT4 vesicles after SDS-PAGE in an effort to observe any changes in the vesicle protein composition, but we were unable to identify any specific changes in the protein profile by this means (Figure 4.7B).

From this data we would suggest that more GLUT4 and IRAP reside in transferrin receptor positive endosomes in prolonged insulin treated cells than in control cells.

4.3.7 GLUT4 is selectively lost from the GSVs in chronic insulin treated cells

As described in chapter 3, iodixanol gradient centrifugation enables us to separate the recycling endosomes from the GLUT4 storage vesicles and it is a powerful method to look at GLUT4 localisation (Hashiramoto & James 2000).

We have used this method to determine whether long term insulin treatment results in an alteration in the pattern of GLUT4 localisation between the two pools. In Figure 4.8 it can be seen that under basal conditions both GLUT4 and IRAP are, as predicted, found in both the GSV and endosomal/TGN peaks. In chronic insulin treated cells on the other hand there is a depletion of both proteins from the GSV peak. In Figure 4.9 the amount of GLUT4 and IRAP present in either peak are expressed as a percentage of the content in control (untreated) cells. The selective loss due to chronic insulin treatment from peak 1 is 67 ± 7% reduction for GLUT4 and 62 ± 6% reduction for IRAP. For peak 2 on the other hand there is a smaller depletion of GLUT4 (19 ± 4.5%), while there is an increase in the amount of IRAP by 38 ± 6%.

In order to be certain that the effect of long term insulin treatment on GLUT4 and IRAP localisation was selective, we examined the distribution of several other membrane proteins. VAMP2, cellubrevin and AP1 (γ-adaptin) did not show any significant change in their distribution between the GSV and the endosomal peak due to chronic insulin treatment (Figure 4.10). This suggests that this treatment does not cause a general alteration to membrane trafficking, but that the effects on GLUT4 and IRAP are specific.

4.4 Discussion

Previous studies have shown that insulin resistance occurs before the onset of diabetes and that the body, in order to overcome this resistance, produces more and more insulin (hyperinsulinaemia) which eventually can lead to the breakdown of insulin secretion and finally to diabetes (as reviewed in Kahn 1994) (Chapter 1.2).

Kozka *et al.* have shown that treatment of 3T3-L1 adipocytes with 500nM insulin for 24 hours, mimicking a hyperinsulinaemic state, decreased the insulin responsiveness of GLUT4 by at least 50% (Kozka *et al.* 1991). In our studies we aimed to determine why insulin ceases to be able to recruit GLUT4 to the plasma membrane under such conditions.

We have confirmed the reduced ability of insulin to mobilise GLUT4 in chronic insulin treated cells using subcellular fractionations, plasma membrane lawn assays and whole cell immunofluorescence. By all these different methods, an acute insulin stimulation could only cause a ~ 1.5-fold stimulation of GLUT4 translocation in

chronic insulin treated cells. We also found that total cellular GLUT4 protein levels decrease due to this treatment, which is in contrast to the study by Kozka *et al.*, but has since been confirmed in many studies, some of which are described below.

The downregulation of GLUT4 in adipocytes by prolonged insulin has been shown to be at the mRNA level (Flores-Riveros *et al.* 1993; MacDougold *et al.* 1995; Hemati *et al.* 1998) as well as at the protein level (Flores-Riveros *et al.* 1993; Sargeant *et al.* 1993), with the half life of GLUT4 decreasing from 50 hours to about 15 hours (Sargeant *et al.* 1993). The decrease of GLUT4 mRNA levels is thought to be due to a dephosphorylation of the transcription factor C/EBPα, which will inactivate the transcription factor and cause a reduction in GLUT4 gene transcription (Hemati *et al.* 1998). It is likely that decreased GLUT4 levels and decreased half life of GLUT4 may also arise as a consequence of GLUT4 being targeted for degradation. Adipocytes from insulin resistant patients have been shown to have a decrease in GLUT4 levels (Garvey *et al.* 1991). Our data therefore is in strong accordance with cells in diabetic patients and supports the use of these cells as a model system.

Many studies have implied that the downregulation of GLUT4 in adipocytes from diabetic patients is the main reason why those cells are insulin resistant. On the other hand, overexpression of GLUT4 in adipocytes using transgenic mice has shown to result in enhanced whole-body insulin sensitivity in both normal as well as diabetic mice (as reviewed in Kahn 1996). This effect was mainly due to increased basal glucose transport explained by an increase of GLUT4 at the plasma membrane. These studies would suggest a more important role of adipose tissue in whole body insulin sensitivity then previously recognised, although adipocytes account for only 5 - 20% of post-prandial glucose disposal (reviewed in Hunter & Garvey 1998). Treatments

that cause upregulation of GLUT4 could therefore greatly increase the insulin sensitivity of diabetic patients. Metformin, an antihyperglycemic agent, has been shown to block the hyperinsulinaemic downregulation of plasma membrane GLUT4 in rat adipocytes (Kozka & Holman 1993) and it is used clinically to alleviate insulin resistance by improving glucose tolerance in type2 diabetic patients (Davidson *et al.* 1997).

If the insulin resistance observed in our study was explained only by a general decrease in GLUT4 levels, we would expect the GLUT4 left in these cells still to be targeted to its special (GSV) compartment and therefore should still show an at least 4-fold translocation to the plasma membrane, although levels in each location compared to control cells should be reduced. Our results do not confirm this hypothesis (Figures 4.1 and 4.2) and we therefore suggest that the GLUT4 left in chronic insulin treated cells is either mis-targeted or that proteins necessary for GLUT4 trafficking are malfunctioning.

To study the second possibility, proteins involved in GLUT4 trafficking were examined.

Interestingly IRAP, which has so far always been thought to behave in a similar manner to GLUT4 (Ross *et al.* 1996; Martin *et al.* 1997), is not down-regulated due to chronic insulin treatment, and its subcellular distribution is also quite different to GLUT4 under these conditions. We found that a larger fraction of IRAP was present in the high density membranes; similar data was observed using other models of insulin resistance, such as high glucose or high glucosamine treatment (Fillipis *et al.* 1998). The authors of this study suggest that this implies a role of IRAP in the

constitutive, but not insulin stimulated GLUT4 translocation (Fillipis *et al.* 1998). Further studies have to be made to confirm a role for IRAP in GLUT4 trafficking. The difference in trafficking between GLUT4 and IRAP found here are thought to be due to different targeting signals in the cytoplasmic domains of those proteins, but so far little is known regarding which sequences may be regulating these events.

Chronic insulin treatment did not change the levels or distribution of the adaptors 1 and 2, and this is of interest as AP1 has been found to co-localise with GLUT4 (Marsh *et al.* 1998) and is even found on GLUT4 vesicles (Gillingham *et al.* 1999). On the other hand, we found that the SNARE proteins VAMP2, syntaxin4 and syndet were upregulated by chronic insulin treatment, and that increased levels of VAMP2 and cellubrevin were now present at the plasma membrane.

Increased SNARE protein levels also occur in muscle of some of the rodent models for insulin resistance (Maier *et al.* 2000). An upregulation of those proteins is in one sense surprising as the reduced GLUT4 translocation observed in these insulin resistant rats would be expected to be accompanied by a decrease in SNARE protein expression. Other studies have reported changes in SNARE protein expression accompanying insulin resistance in pancreatic beta-cells. Thus, in *fa/fa* as well as in the GK rats (both rodent models for type II diabetes), insulin secretion due to high glucose is impaired and in both studies a down-regulation of some SNARE proteins was found (Chan *et al.* 1999; Nagamatsu *et al.* 1999). Restoration of the SNARE protein levels in GK rats improved glucose-stimulated insulin secretion. However, and interestingly from the perspective of the data described here, overexpression of the SNARE proteins in normal rat islets decreased glucose-stimulated insulin release

(Nagamatsu *et al.* 1999), suggesting that over-expression of SNARE proteins can profoundly alter membrane trafficking events.

Upregulation of key proteins for GLUT4 trafficking could be a means of compensating for the reduced glucose uptake. Although it is not clear whether the changes in SNARE protein expression are causal or adaptive, we suggest that normal levels of SNARE proteins are important for their correct function in the cell. So far no one to our knowledge has looked at SNARE proteins involved in GLUT4 trafficking in insulin resistance and we show here for the first time that the insulin resistant state causes alterations in key trafficking proteins.

In order to study the possibility that GLUT4 is mis-targeted in chronic insulin treated cells several different methods have been applied to examine the cellular localisation of GLUT4 in those cells.

The method of transferrin receptor ablation has been commonly used in order to distinguish between the GLUT4 in the recycling endosomal system and GLUT4 in the GSVs (Livingstone *et al.* 1996). The increase in transferrin receptor ablation due to chronic insulin treatment could mean that the TfR is now more susceptible for Tf-HRP binding due to its increased recycling in the constant presence of insulin. Similarly, the increased ablation of IRAP suggests a higher proportion of this protein is in a TfR positive compartment. Ablation results do not show an increase of GLUT4 in the ablatable pool with the amount of ablation being similar to control cells, but this is in contrast to the results using vesicle adsorption where there seems to be an increased co-localisation of GLUT4 with the TfR. Even in control cells we can not detect the 40% ablation of GLUT4 reported (Livingstone *et al.* 1996) and this

might be the reason why there is no difference in the treated cells. However, it is important to recognise that the vesicle adsorption approach cannot distinguish between vesicles containing different ratio's of TfR to GLUT4. Hence, vesicles containing an average of 1 molecule of GLUT4 and 10 molecules of TfR (such as control cells) and vesicles containing 1 molecule of GLUT4 and 30 molecules of TfR (such as chronic insulin treated cells) are equally bound by the protein A-anti-GLUT4 conjugate. The apparent increase in TfR content of GLUT4 vesicles therefore does not necessarily mean that more GLUT4 is localised to TfR-positive endosomes, but may simply reflect more TfR present in endosomes under chronic insulin treated conditions. This is confirmed by the fact that more TfR is ablated in chronic insulin treated cells than control cells and suggests that more TfR moves to the endosomes from deeper less accessible compartments in the cell. The crucial result here is the increased IRAP ablation observed in chronic insulin treated conditions, which implies that the extent of overlap of IRAP and TfR has increased and that therefore more IRAP is localised to endosomes than in control cells.

To further clarify this we used iodixanol gradient centrifugation, as this method has greatly helped to study the different GLUT4 compartments. We have found here that in chronic insulin treated cells the cellular depletion of GLUT4 is selectively from the GSVs and that no more GLUT4 gets sorted into the GSVs. The results for IRAP on the gradient look similar to GLUT4 but, as mentioned earlier, total cellular IRAP levels do not change due to chronic insulin treatment and is therefore a redistribution of IRAP from the GSVs to the plasma membrane (Figure 4.1) and to the endosomes in these cells (Figure 4.8).

GLUT4 on the other hand is downregulated by about 40% due to prolonged insulin treatment (Figure 4.3.B) and very little GLUT4 is present at the plasma membrane (Figure 4.1A). This data therefore shows that in these cells there is a selective loss of GLUT4 from the GSVs and the majority of the GLUT4 still present is now localised to the endosomes.

Together these results support the hypothesis that GLUT4 is mis-targeted in chronic insulin treated cells (Figure 4.11). We propose that prolonged insulin causes the translocation of nearly all GLUT4 and IRAP to the plasma membrane. From there these proteins are endocytosed and are taken into the recycling endosomes. At this stage there seems to be a block in the sorting to the GSVs, supported by the low levels of both IRAP and GLUT4 in the GSV peak (Figure 4.8), and GLUT4 is presumably taken to a large part into lysosomes for degradation, confirmed by the downregulation of GLUT4 (Figure 4.3B). IRAP on the other hand stays in the endosomes (Figure 4.9) and this is supported by an increased co-localisation of the TfR with IRAP (Figures 4.6 and 4.7). In addition, IRAP translocation to the plasma membrane due to insulin is now about 1.5 - 2-fold (Figure 4.2B), which corresponds to the insulin sensitivity of the endosomes.

In the cascade of events from insulin distribution in the blood to the effect it has on muscle and fat cells and to the final uptake of glucose into the cells there are many possible sites of defect which all could lead to a insulin resistant phenotype. The effects of long term insulin treatment have also been looked at from a signalling perspective. It is known that chronic insulin will also cause changes in many signalling proteins such as the insulin receptor and IRS-1 (Knutson *et al.* 1995; Ricort *et al.* 1995; Pryor *et al.* 2000). Chronic insulin causes downregulation of IRS-

1 and IRS-2 in 3T3-L1 adipocytes (Clark *et al.* 2000). A study in cultured rat adipocytes showed that chronic insulin treatment causes a decreased tyrosine phosphorylation of the insulin receptor β-subunit as well as several other changes in the signalling cascade, but this did not lead to a change of the exocytosis rate of GLUT4 (Pryor *et al.* 2000). Similar changes in signalling proteins have also been found in diabetic patients. Some patients were found to have abnormalities in insulin receptors, however a defect for example in the intrinsic tyrosine kinase activity of the receptor can be reversed through weight loss and euglycemic therapy. (Freidenberg *et al.* 1988; Freidenberg *et al.* 1991). This illustrates that defects like this might be secondary rather then the cause of insulin resistance. It is now known that the major downstream event which seems to be defective in all insulin resistant patients is the translocation of GLUT4 from an intracellular store to the plasma membrane (as reviewed in Hunter & Garvey 1998). From our results this would therefore suggest that mis-targeting of GLUT4 is the main reason for insulin resistance. Interestingly mis-targeting of GLUT4 has also been suggested in muscle from type 2 diabetic patients (Chapter 1.5.5), but due to the difficulties in fractionating muscle it is not clear from this study (Garvey *et al.* 1998) where GLUT4 is found and whether this accounts for the decrease in glucose transport.

In order to examine whether GLUT4 is selectively lost from the GSV compartment in insulin resistant patients it would be of great interest to look at the distribution of GLUT4 in muscle and fat. We have established that GLUT4, using human fat tissue, can be separated into two pools using iodixanol gradient centrifugation (data not shown), but due to the small amount of material obtainable and the small number of samples, we have not yet been able to see any differences in GLUT4 distribution in healthy versus diabetic patients. Further studies to look at this are under way.

In conclusion we have shown here that prolonged insulin treatment of 3T3-L1 adipocytes renders these cells insulin resistant. This is accompanied by a downregulation of GLUT4 and an upregulation of different SNARE proteins. The loss of GLUT4 from these cells is selective from the GSV compartment and this can explain the diminished GLUT4 fold translocation due to insulin in these cells. Finally these data illustrate that, under our experimental conditions, the trafficking of IRAP and GLUT4 are distinct.

Figure 4.1A Subcellular fractionation of 3T3-L1 adipocytes and the effect of chronic insulin treatment on GLUT4 and IRAP distribution

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated with 1μM insulin for 30 minutes. Subcellular fractions were prepared as described in *Materials and Methods (section 2.11.1)*. 20μg of protein was separated by SDS-PAGE and immunoblotted with the antibodies indicated. The resulting membrane fractions were plasma membrane (PM), low density membranes (LDM) and high density membranes (HDM). Shown is a representative immunoblot and similar results were obtained from at least three experiments.

Figure 4.1A

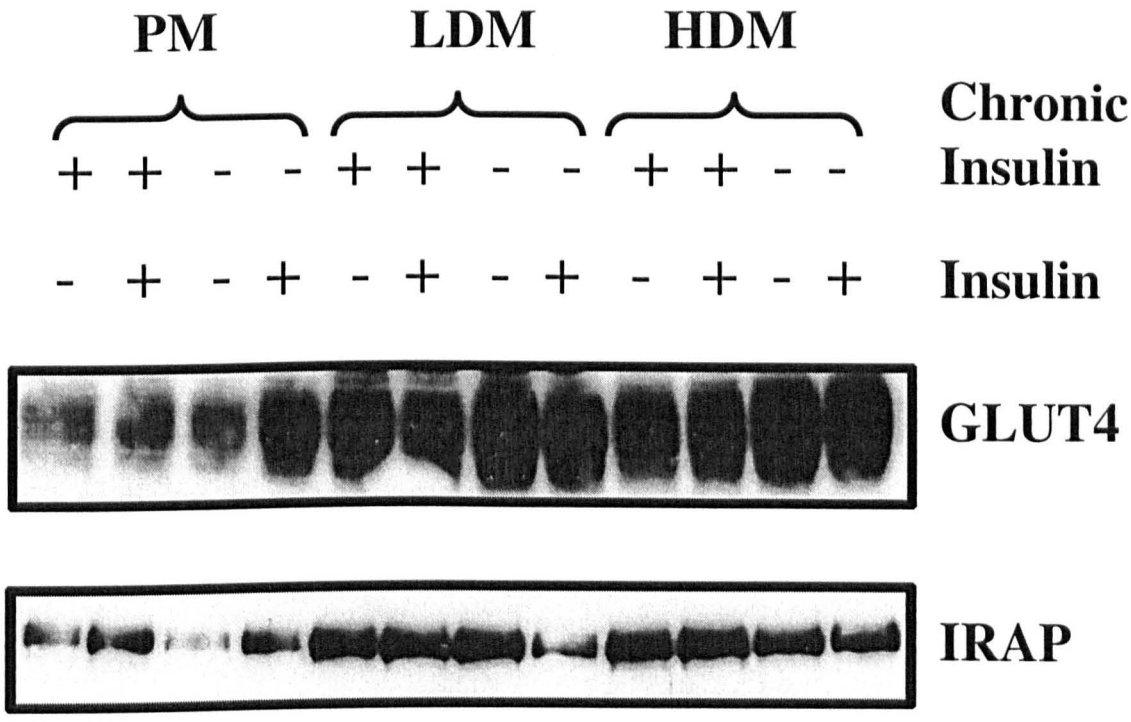


Figure 4.1B Subcellular fractionation of 3T3-L1 adipocytes and the effect of chronic insulin treatment on the protein distribution

3T3-L1 adipocytes were treated with (CI) or without (B) insulin at 500nM for 24 hours and washed as outlined in *Materials and Methods (section 2.14).* Subcellular fractions were prepared as described in *Materials and Methods (section 2.11.1)* and analysed by SDS-PAGE. The resulting membrane fractions were plasma membrane (PM), low density membranes (LDM), high density membranes (HDM), and soluble proteins (SP). Shown is a silver nitrate stained gel of such an experiment.

Figure 4.1B

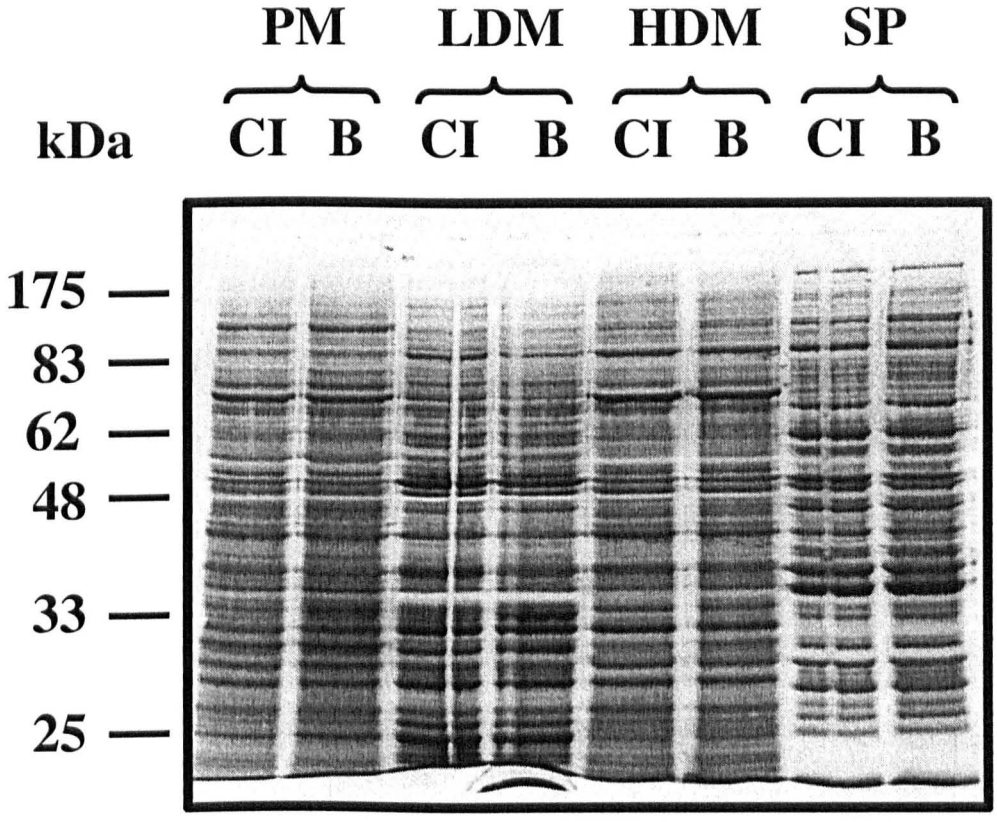
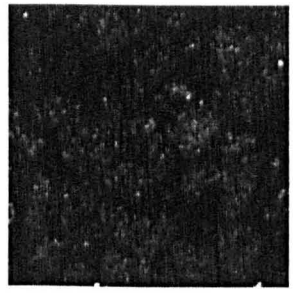


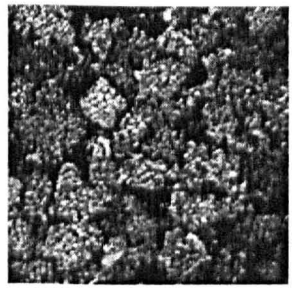
Figure 4.2A Plasma Membrane Lawn assay of insulin-stimulated GLUT4 translocation

3T3-L1 adipocytes grown on glass cover-slips were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated with 1μM insulin for 30 minutes and plasma membrane lawns were prepared as described in *Materials and Methods (section 2.13.1)* and stained with GLUT4 antibody. Shown are the results of a typical experiment (n=3) in which representative fields are shown.

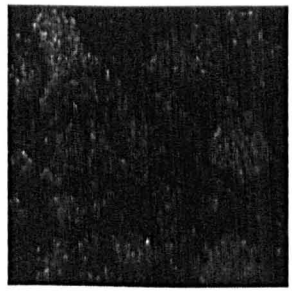
Figure 4.2A



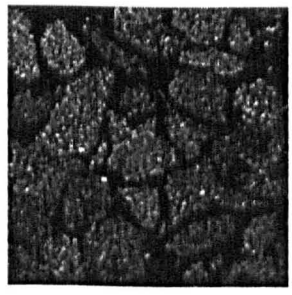
Basal



Insulin



Chronic Insulin



Chronic Insulin + Insulin

Figure 4.2B Insulin-stimulated GLUT4 and IRAP translocation differs in chronically insulin treated 3T3-L1 adipocytes

3T3-L1 adipocytes grown on glass cover-slips were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated with 1μM insulin for 30 minutes and the extent of GLUT4 and IRAP translocation determined using the plasma membrane lawn technique *Materials and Methods (section 2.13.1)*. Shown is the fold translocation over basal from two (IRAP) and four (GLUT4) experiments. For each condition at least 4 different areas of the coverslip were scanned and quantified. The mean of those was used to calculate the fold increase at the plasma membrane. B = basal cells, B+I = control cells acutely stimulated with insulin. CI = chronic insulin treated cells, and CI+I = chronic insulin treated cells acutely challenged with insulin.* indicates a significant difference increase in cell surface levels of IRAP compared to basal cells (B), p=0.02. ** indicates a significant reduction in insulin-stimulated GLUT4 translocation compared to B+I, $p<0.01$, and † indicates a significant reduction in insulin-stimulated IRAP translocation compared to B+I, $p<0.05$.

Figure 4.2B

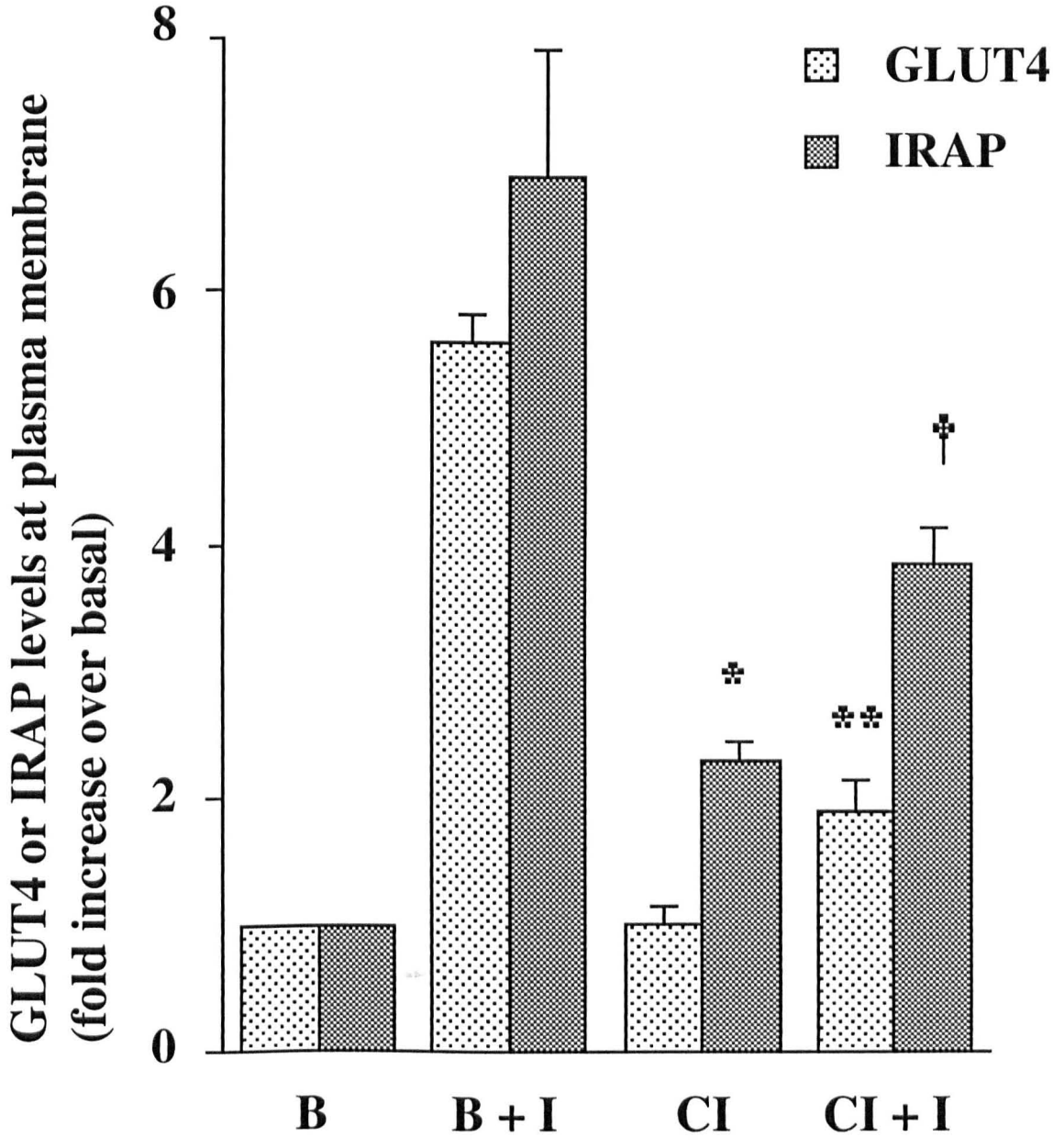
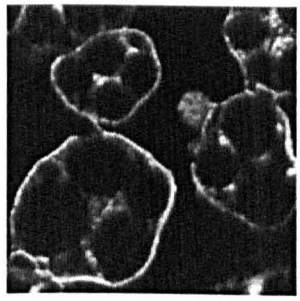

GLUT4 or IRAP levels at plasma membrane
(fold increase over basal)
8
6
4
2
0
GLUT4
IRAP
B
B + I
CI
CI + I
*
**
†

Figure 4.2C Whole cell Immunofluorescence of GLUT4 distribution in 3T3-L1 adipocytes

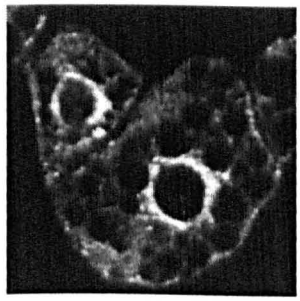
3T3-L1 adipocytes grown on glass cover-slips were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated or not with 1μM insulin for 30 minutes and cells were stained with GLUT4 antibody as described in *Materials and Methods (section 2.13.2).* Shown are representative images from one experiment.

Figure 4.2C

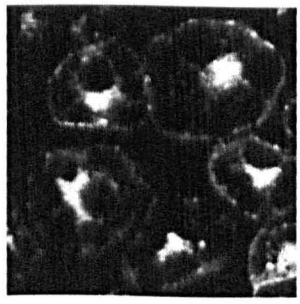
Basal



Insulin



Chronic Insulin



Chronic Insulin + Insulin

Figure 4.3A Analysis of protein expression levels in chronically insulin treated 3T3-L1 adipocytes

Total membranes from control and chronically insulin treated 3T3-L1 adipocytes were prepared as outlined in *Materials and Methods (section 2.11.2).* Shown are representative immunoblots of the indicated amounts of membrane protein probed using the antibodies indicated.

Figure 4.3A

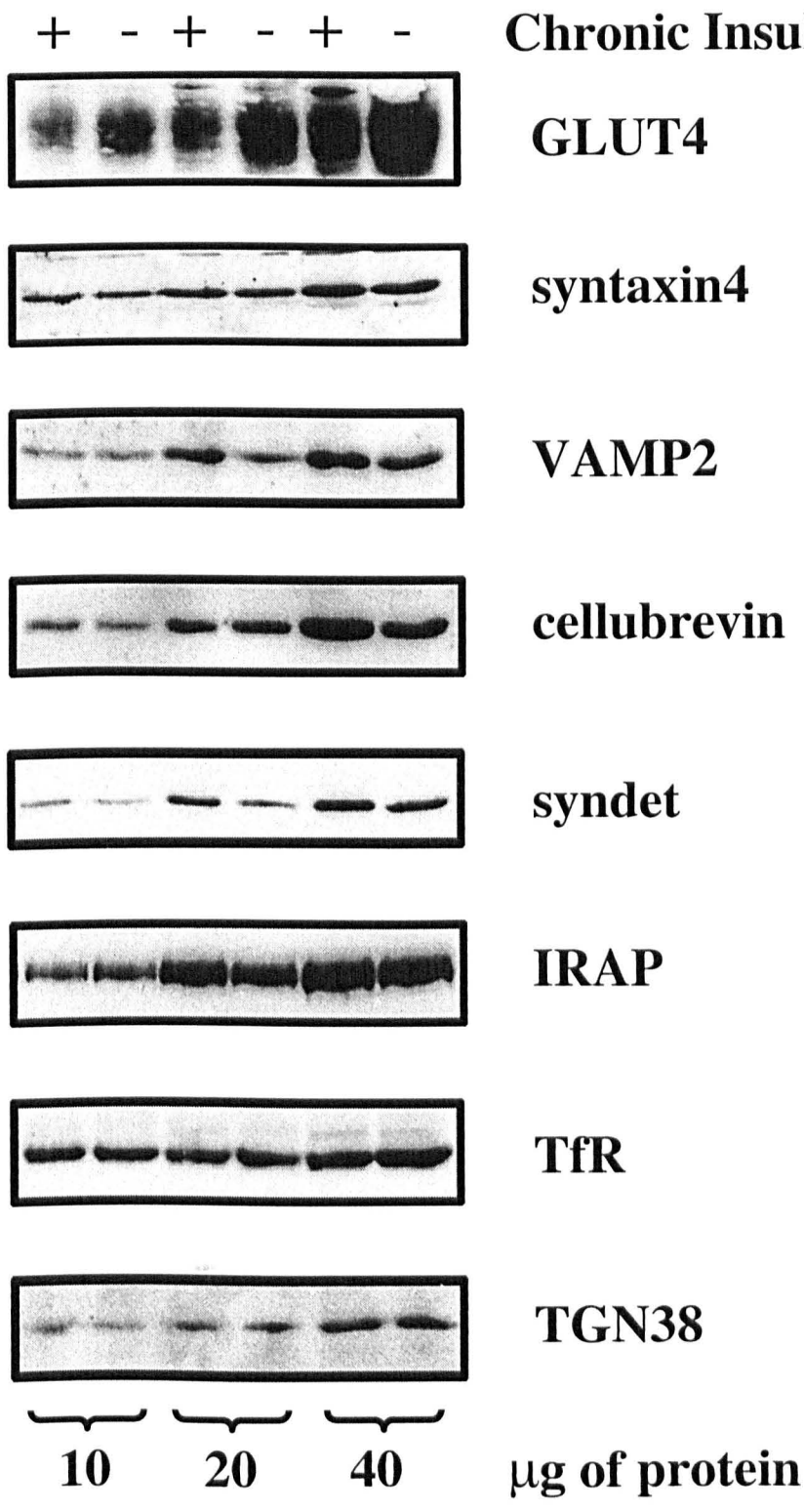

+ - + - + -
Chronic Insulin
GLUT4
syntaxin4
VAMP2
cellubrevin
syndet
IRAP
TfR
TGN38
10
20
40
μg of protein

Figure 4.3B Quantification of protein expression levels in chronically insulin treated 3T3-L1 adipocytes

Total membranes from control and chronically insulin treated 3T3-L1 adipocytes were prepared as outlined in *Materials and Methods (section 2.11.2)*. Samples were separated by SDS-PAGE and immunoblotted with respective antibodies. Shown is the quantification of this data (mean ± s.e.m. from three independent preparations of membranes). In this analysis, protein levels in control cells are ascribed a value of 100% and the effect of chronic insulin treatment on protein levels is expressed as a % change from this value. Significant differences from control cells not exposed to chronic insulin treatment are indicated by *$p<0.05$, **$p=0.02$, ***not significant, ****$p=0.04$ and $^{\dagger}p<0.05$.

Figure 4.3B

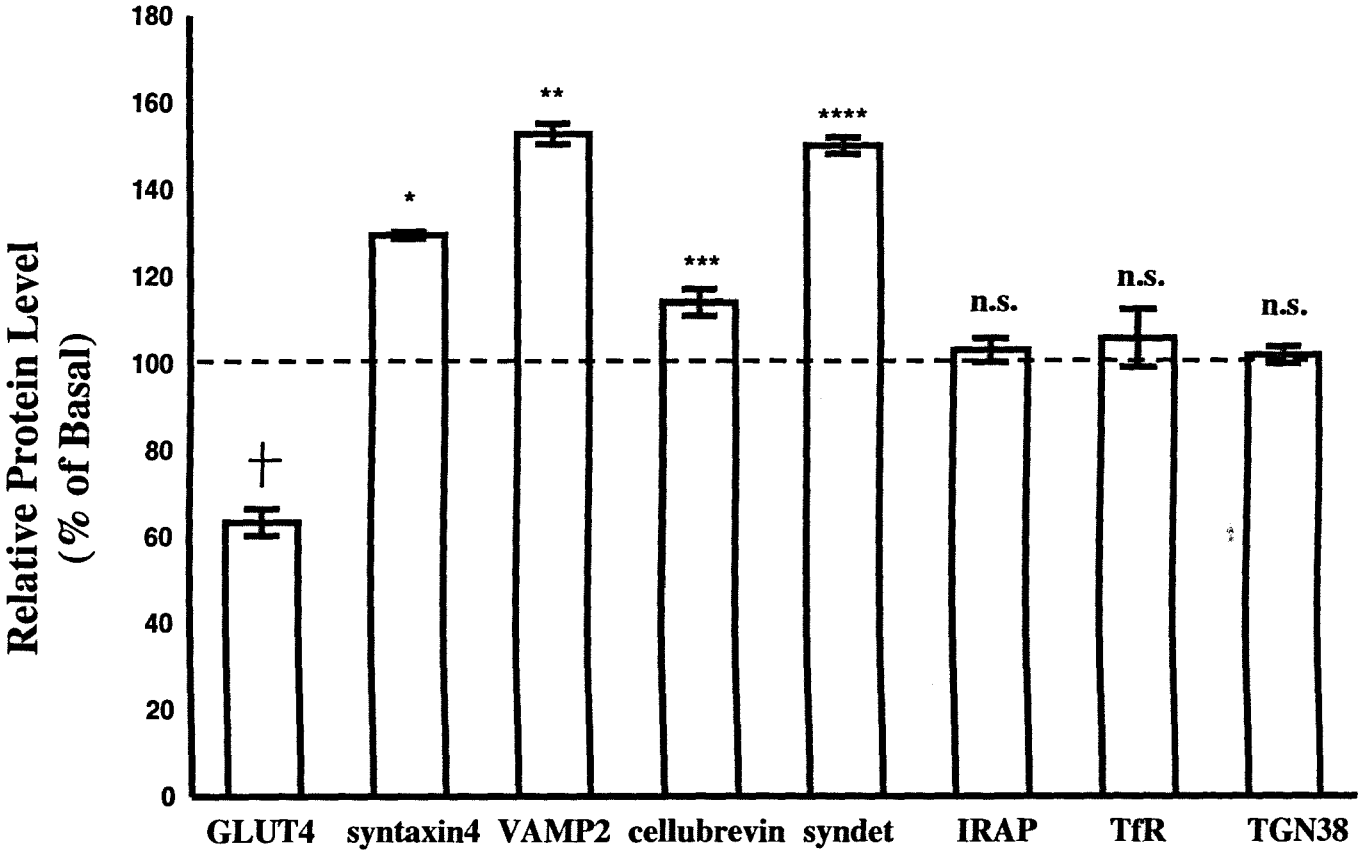

Relative Protein Level
(% of Basal)
180
160
140
120
100
80
60
40
20
0
*
**
***
****
n.s.
n.s.
n.s.
GLUT4
syntaxin4
VAMP2
cellubrevin
syndet
IRAP
TfR
TGN38

Figure 4.4 Subcellular fractionation of 3T3-L1 adipocytes and the effect of chronic insulin treatment on the SNARE proteins

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated with 1μM insulin for 30 minutes. Subcellular fractions were prepared as described in *Materials and Methods (section 2.11.1)* 20μg of protein was separated by SDS-PAGE and immunoblotted with the antibodies indicated. The resulting membrane fractions were plasma membrane (PM), low density membranes (LDM) and high density membranes (HDM). Shown is a representative immunoblot and similar results were obtained from three experiments.

Figure 4.4

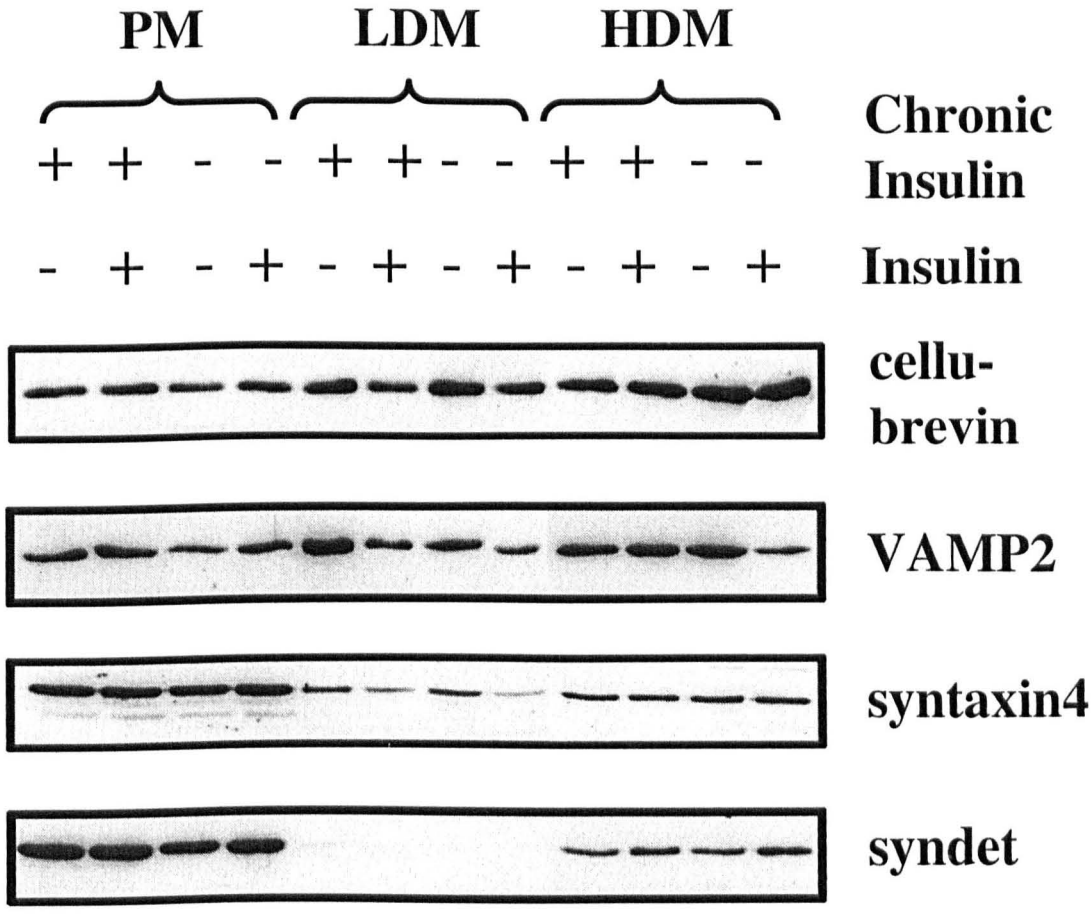

PM
LDM
HDM
+ + - - + + - - + + - -
Chronic Insulin
- + - + - + - + - + - +
Insulin
cellu-brevin
VAMP2
syntaxin4
syndet

Figure 4.5 Chronic insulin treatment causes no change in AP1 and AP2 adaptor distribution

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were acutely insulin-stimulated with 1μM insulin for 30 minutes. Subcellular fractions were prepared as described in *Materials and Methods (section 2.11.1)*. 20μg of protein was separated by SDS-PAGE and immunoblotted with the antibodies indicated. The resulting membrane fractions were plasma membrane (PM), low density membranes (LDM), high density membranes (HDM) and soluble proteins (SP). Shown is a representative immunoblot, similar results were obtained from two experiments.

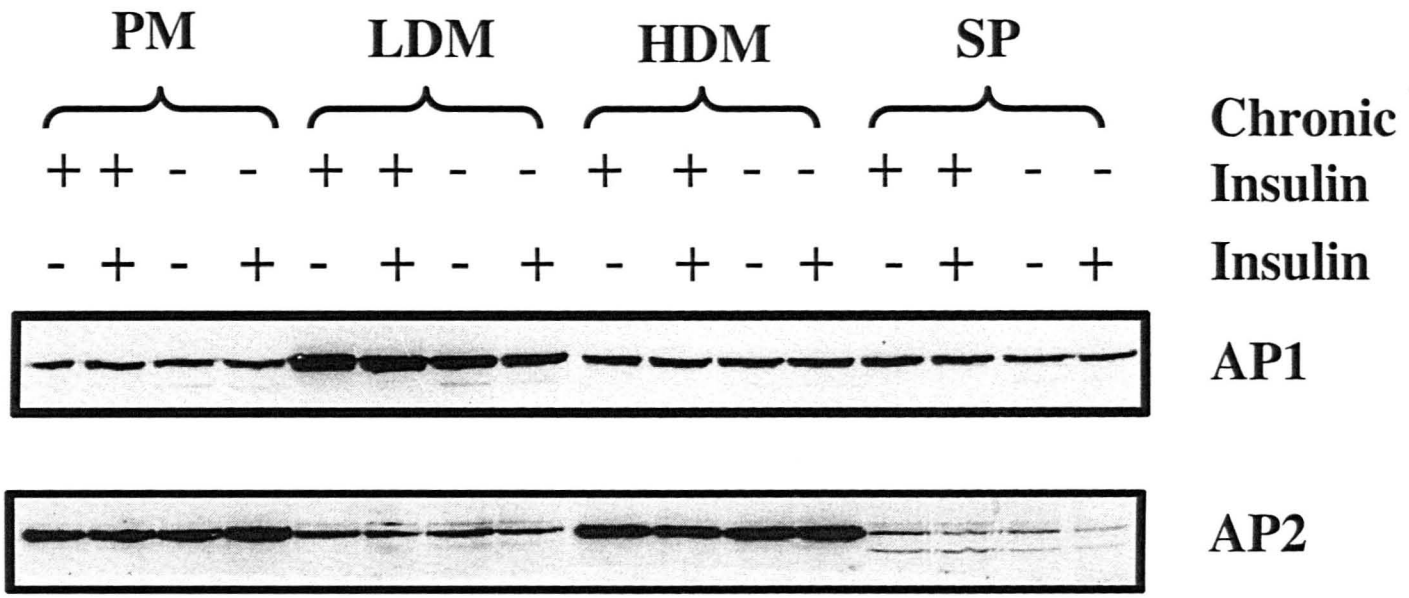


Figure 4.5

Figure 4.6 Ablation of chronically insulin stimulated 3T3-L1 adipocytes

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)*, cells were incubated with TfR-HRP for 1 hour and ablation was performed as described in *Materials and Methods (section 2.10.2).* LDM's were prepared and 20μg of protein separated by SDS-PAGE. Shown are typical immunoblots for the antibodies indicated (n=3 for GLUT4, n=2 for Transferrin Receptor and IRAP). H_2O_2 indicates ablation.

Figure 4.6

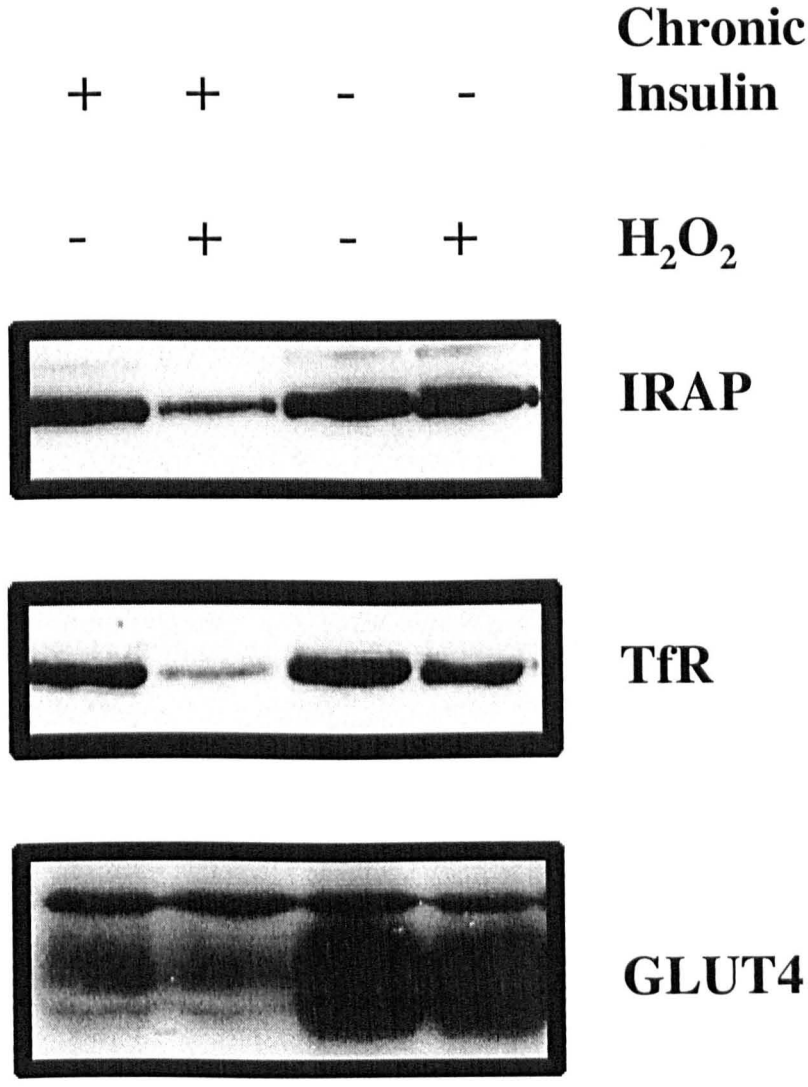


Figure 4.7A The effect of chronic insulin treatment on GLUT4 vesicle adsorption

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)* LDM's were prepared and used for immunoadsorption of vesicles, with either GLUT4 antibody or a random IgG as control (IP AB), as described in *Materials and Methods (section 2.12)*. One fifth of the vesicle and LDM fractions were then separated by SDS-PAGE and immunoblotted with the antibodies indicated (IB AB). Shown are immunoblots from a single experiment.

Figure 4.7A

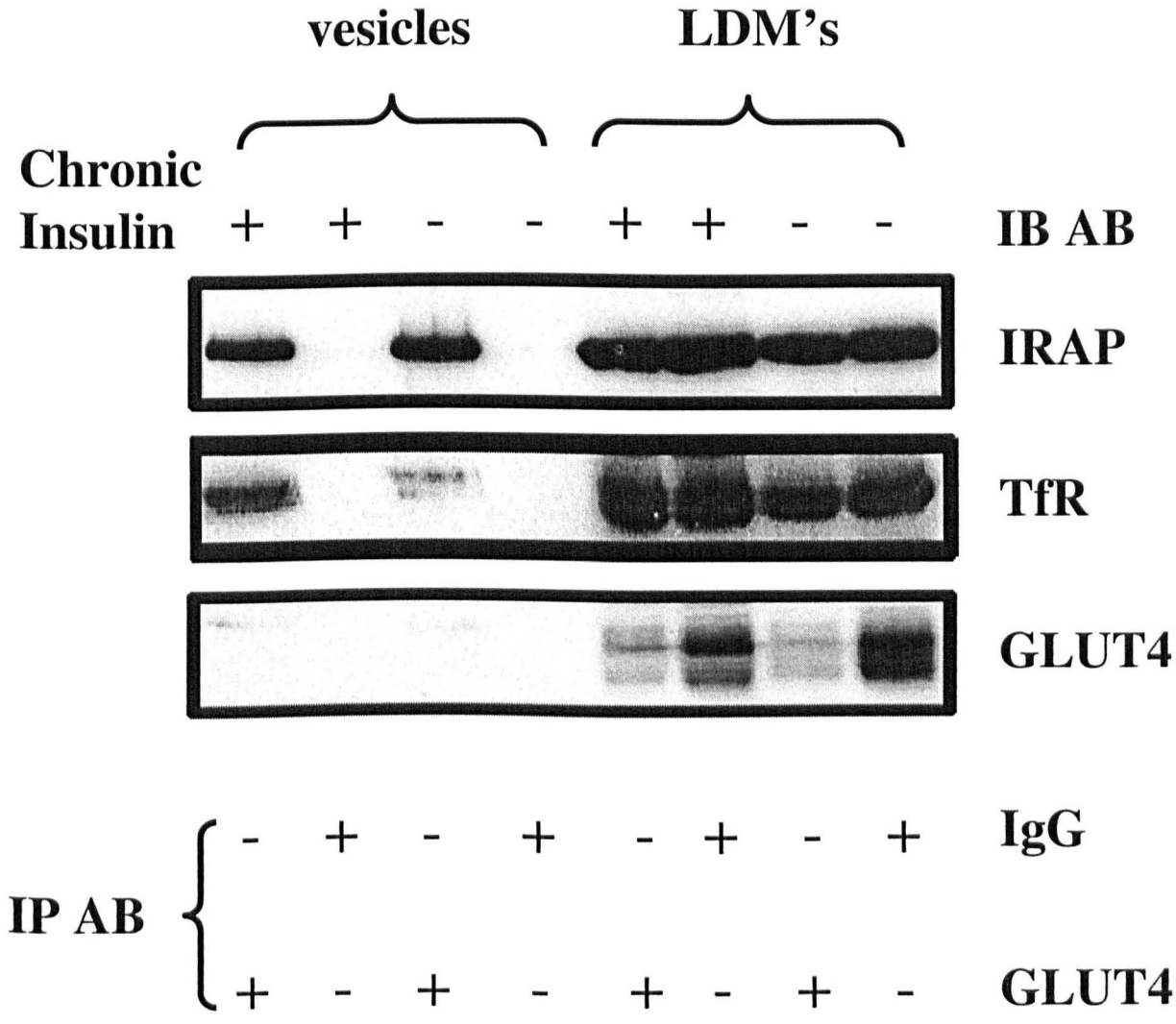

vesicles
LDM's
Chronic Insulin
+ + - - + + - -
IB AB
IRAP
TfR
GLUT4
IP AB
- + - + - + - + IgG
+ - + - + - + - GLUT4

Figure 4.7B The effect of chronic insulin treatment on GLUT4 vesicle protein profiles

3T3-L1 adipocytes were treated with or without insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)* LDM's were prepared and used for immunoadsorption of vesicles, with either GLUT4 antibody or a random IgG as control, as described in *Materials and Methods (section 2.12).* One fifth of the vesicle and 1/20th of LDM fractions were then analysed by SDS-PAGE and the gel was stained with silver nitrate. Shown is a gel from a representative experiment.

Figure 4.7B

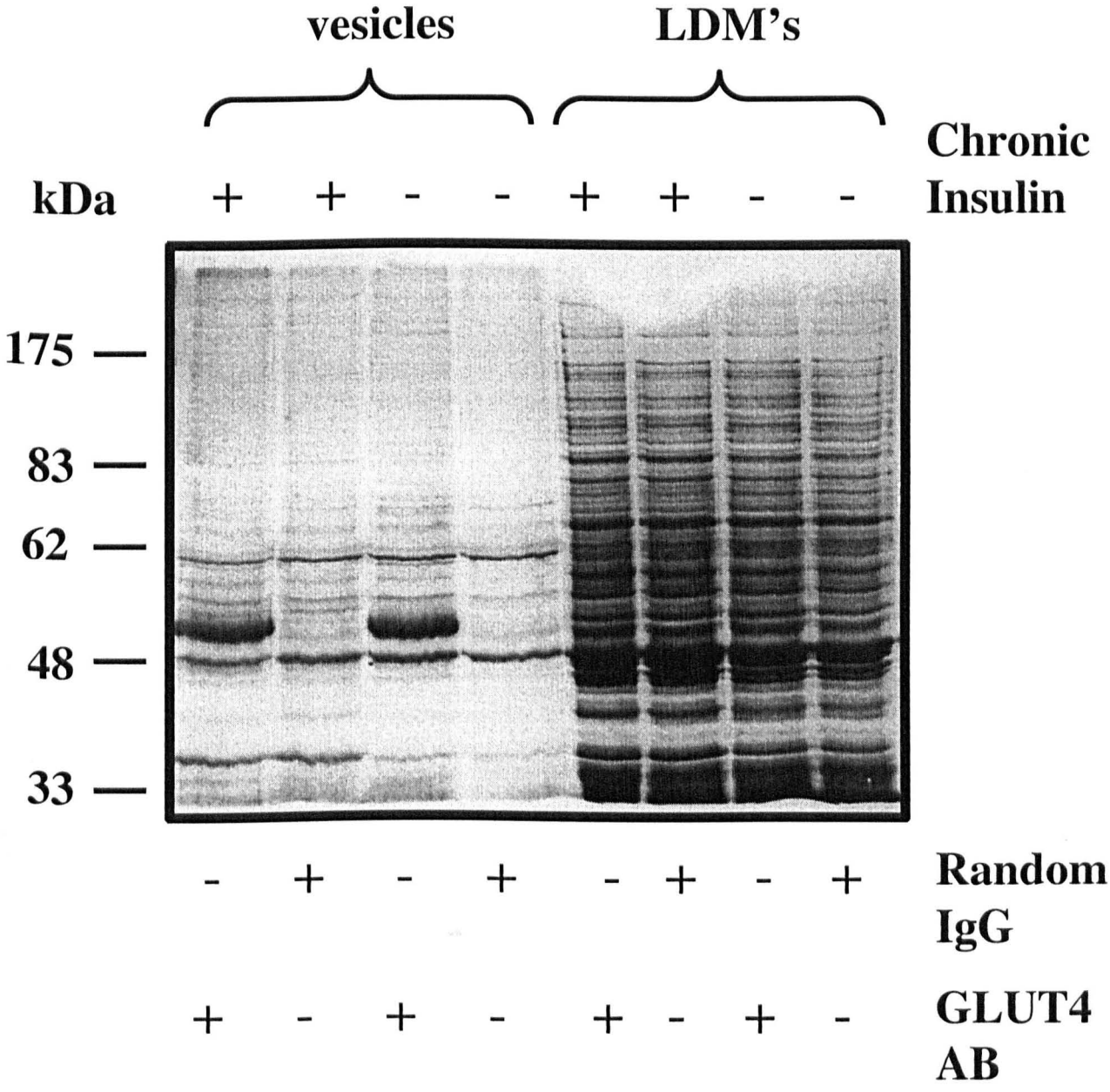

vesicles
LDM's
Chronic Insulin
kDa
+ + - - + + - -
175
83
62
48
33
- + - + - + - +
Random IgG
+ - + - + - + -
GLUT4 AB

Figure 4.8 Iodixanol gradient sedimentation analysis of GLUT4 and IRAP in 3T3-L1 adipocytes and the effect of chronic insulin treatment

3T3-L1 adipocytes were treated with (CI) or without (B) insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)* LDM fractions were prepared and subjected to iodixanol density gradient analysis *Materials and Methods (section 2.15).* Fractions were collected from the bottom of the gradient and ca. 1/10 of each fraction was separated by SDS-PAGE. A typical immunoblot for GLUT4 and IRAP is shown.

Figure 4.8

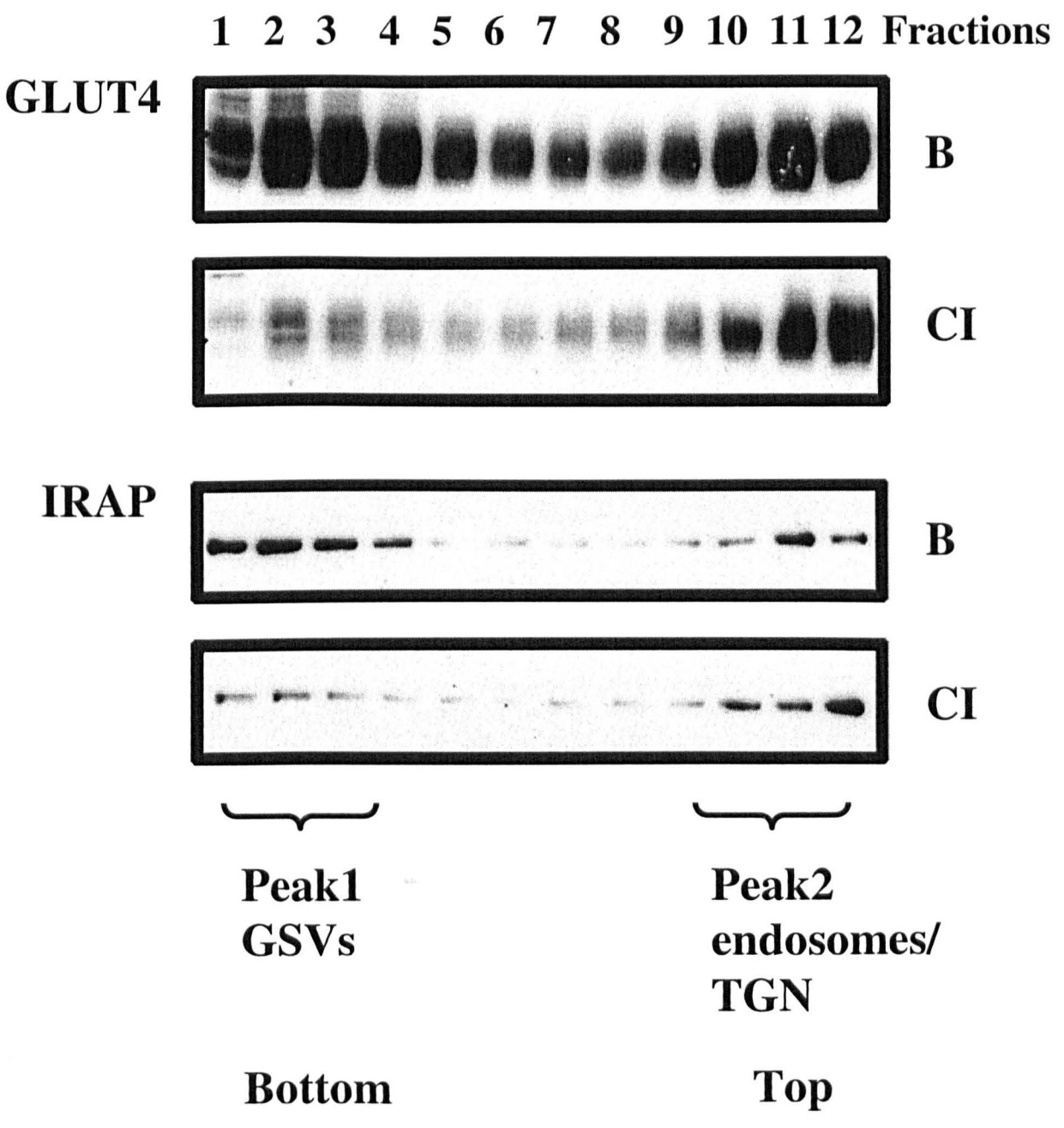

1 2 3 4 5 6 7 8 9 10 11 12 Fractions
GLUT4
B
CI
IRAP
B
CI
Peak1
GSVs
Peak2
endosomes/
TGN
Bottom
Top

Figure 4.9 Quantitation of Iodixanol gradient sedimentation analysis of GLUT4 and IRAP in 3T3-L1 adipocytes and the effect of chronic insulin treatment

3T3-L1 adipocytes were treated with (CI) or without (B) insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)* LDM fractions were prepared and subjected to iodixanol density gradient analysis. Collected fractions were separated by SDS-PAGE and immunoblots from four different experiments were quantified. Shown is the % change in GLUT4 and IRAP in each of the two peaks. The reduction in GLUT4 and IRAP content of peak 1 were both statistically significant (*$p<0.02$ in both cases). The elevated level of IRAP in peak 2 in chronic insulin cells was statistically significant compared to basal cells ($^{\dagger}p=0.04$).

Figure 4.9

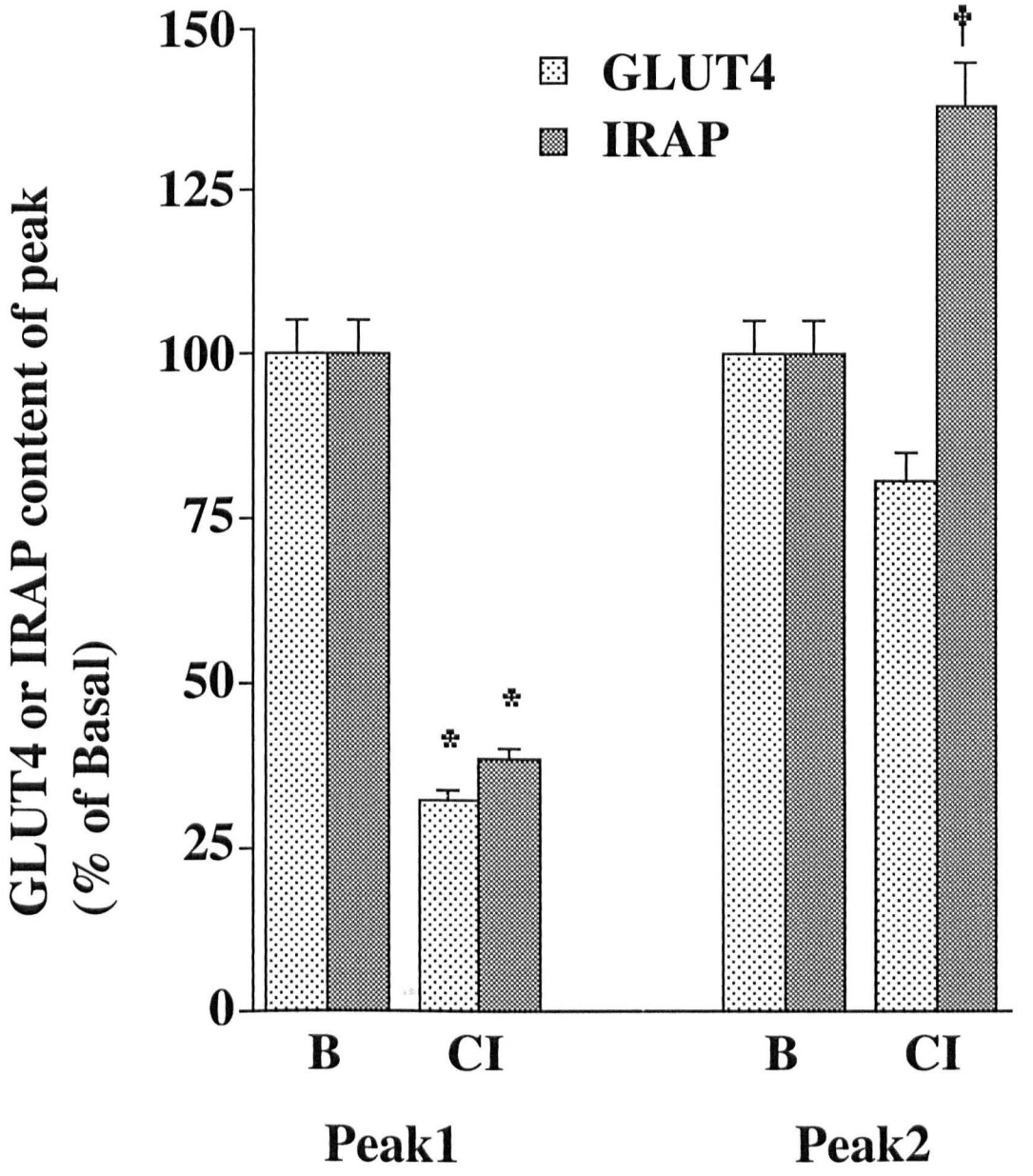

GLUT4
IRAP
GLUT4 or IRAP content of peak
(% of Basal)
150
125
100
75
50
25
0
B
CI
B
CI
Peak1
Peak2

Figure 4.10 Iodixanol gradient sedimentation analysis of VAMP2, cellubrevin and AP1 in 3T3-L1 adipocytes and the effect of chronic insulin treatment

3T3-L1 adipocytes were treated with (CI) or without (B) insulin at 500nM for 24 hours. After washing as outlined in *Materials and Methods (section 2.14)* LDM fractions were prepared and subjected to iodixanol density gradient analysis as described earlier. Fractions were collected from the bottom of the gradient and ca. 1/10 of each fraction was separated by SDS-PAGE, and immunoblotted with the antibodies indicated.

Figure 4.10

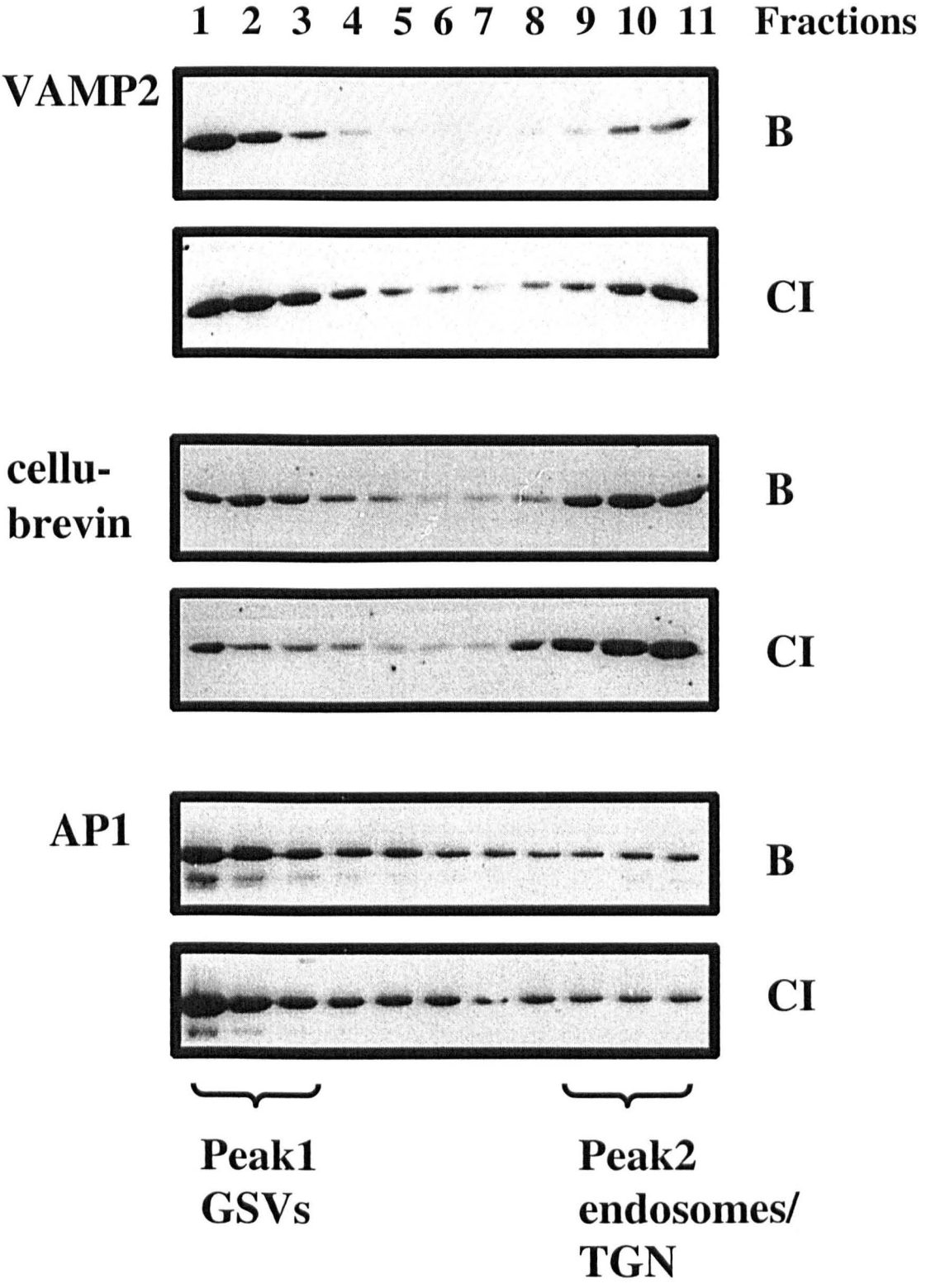
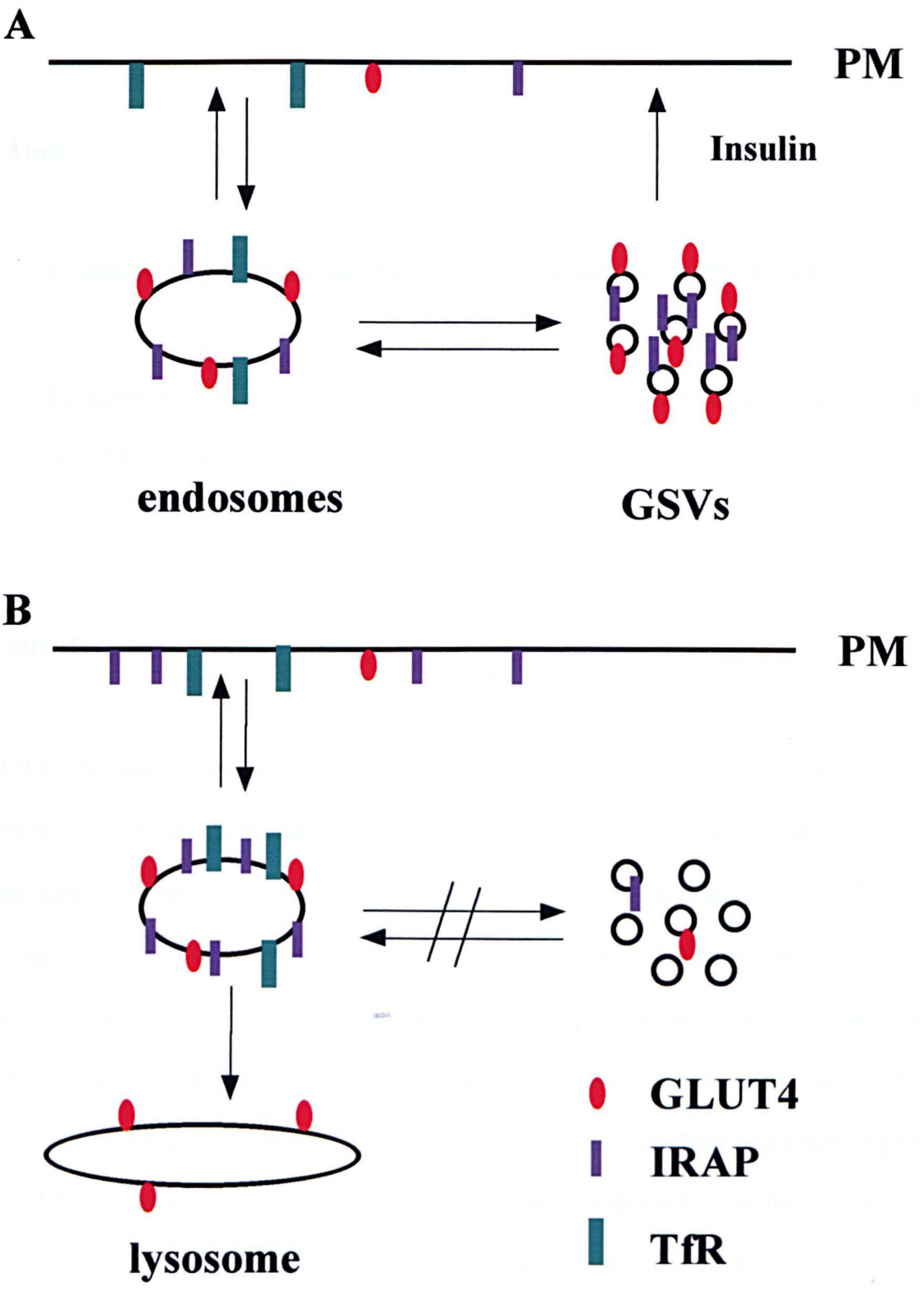

1 2 3 4 5 6 7 8 9 10 11 Fractions
VAMP2
B
CI
cellu-
brevin
B
CI
AP1
B
CI
Peak1
GSVs
Peak2
endosomes/
TGN

Figure 4.11 Model for the distribution of several membrane proteins in chronic insulin treated cells

A In control cells in the absence of insulin about 60% of GLUT4 are localised in the GSV compartment which is highly insulin sensitive. 40% of GLUT4 are found in the endosomes together with the Transferrin Receptor. IRAP shows a very similar distribution to GLUT4. Both proteins are virtually absent from the plasma membrane in the absence of insulin.

B In cells that were treated for a prolonged period of time with insulin GLUT4 cellular levels are downregulated probably by targeting GLUT4 to the lysosomes. There is a block in the targeting of proteins into the GSVs and proteins accumulate in the endosomes. IRAP and TfR co-localise to a higher degree under these conditions.

Figure 4.11



CHAPTER 5 - CHARACTERISATION OF CLATHRIN ADAPTORS IN 3T3-L1 ADIPOCYTES AND THEIR INTERACTION WITH GLUT4 AND IRAP

5.1 Aims

1. To determine the cellular distribution of clathrin adaptors in 3T3-L1 adipocytes

2. To examine whether there is an interaction between these adaptors and either GLUT4 or IRAP

5.2 Introduction

GLUT4, the insulin responsive glucose transporter, is intracellularly localised in the absence of insulin and is thought to be distributed ~40% in the endosomes and ~60% in a specialised insulin sensitive compartment called GLUT4 storage vesicles (GSVs) (Livingstone *et al.* 1996; Kandror 1999 and reviewed in Rea & James 1997). GLUT1, which has a much wider tissue distribution then GLUT4 (reviewed in Gould & Holman 1993), co-localises in part with GLUT4 in adipocytes, but is found partly at the cell surface (Calderhead *et al.* 1990). GLUT1 is also insulin sensitive, but to a lesser degree then GLUT4. It is now thought that GLUT1 co-localises with GLUT4 in the endosomes and recycles constantly between this compartment and the plasma membrane, while GLUT4 is sequestered into GSVs (as reviewed in Rea & James 1997). These differences

in targeting of the two transporters have led many investigators to examine the amino acid sequences of the cytosolic domains of the transporters, as differences in the sequences of these regions might explain why GLUT1 and GLUT4 behave differently. It has been found, as described below, that the N- and C terminal tails of GLUT4 are especially important for its localisation.

The N-terminus of GLUT4 contains a motif (F^5QQI^8) which resembles the tyrosine-based internalisation motifs found in other membrane proteins such as the transferrin receptor, TGN38 and the mannose 6-phosphate receptors (as reviewed in Bonifacino & Dell'Angelica 1999). This motif is thought to be important for the internalisation of the membrane protein as well as for targeting from endosomes to lysosomes and other compartments (reviewed in Marks *et al.* 1997; Bonifacino & Dell'Angelica 1999). In GLUT4, mutation of the phenylalanine 5 to alanine results in increased levels of the transporter at the plasma membrane and endosomes, indicating the importance of this motif for internalisation (Piper *et al.* 1993) and possible targeting of GLUT4 to the GSVs or TGN (Melvin *et al.* 1999).

The GLUT4 C-terminus has been implied to be important in intracellular retention of GLUT4 (Lee *et al.* 1997). The C-terminus of GLUT4 contains a di-leucine motif and such motifs have also been found to be important for endocytosis in other membrane proteins (Marks *et al.* 1997). In 3T3-L1 adipocytes mutational studies of the di-leucine motif in GLUT4 have highlighted the importance of this motif in internalisation (Verhey *et al.* 1995) as well as a possible role in the trafficking of GLUT4 from the TGN to the endosomes (Melvin *et al.* 1999). Additionally, other residues within the C-terminus of GLUT4 (distinct from the di-leucine motif) are also known to be important in targeting

GLUT4 (Verhey *et al.* 1995; Sandoval *et al.* 2000). The acidic cluster at the extreme C-terminus of GLUT4 (Shewan *et al.* 2000) as well as two glutamate residues adjacent to the di-leucine motif (Cope *et al.* 2000) have been shown to be crucial for targeting GLUT4 to the GSVs.

Endocytosis and intracellular targeting of membrane proteins is known to occur via the recruitment of proteins such as clathrin and the clathrin adaptors to the membrane. These adaptors mediate association of clathrin with the membrane by recognising and binding to the different motifs in the membrane protein. This is best understood for clathrin-mediated endocytosis, where the adaptor AP2 binds the membrane protein to be internalised, resulting in the recruitment of clathrin to the membrane (as reviewed in Robinson 1994; Bonifacino & Dell'Angelica 1999). Clathrin is a scaffold protein and often called a triskelion due to its three legged structure. It is made of three large 190kDa subunits and three small subunits around 25kDa in size. The crystal structure of the N-terminus of the large subunit has greatly helped in studying the assembly of clathrin and its function in the recruitment of cargo (ter Haar *et al.* 1998). Adaptors can bind clathrin and stimulate the formation of clathrin cages. Thereby adaptors link the coat formation to cargo incorporation and prepare the membranes for endocytosis. Clathrin forming a lattice causes the invagination of the membrane and with the help of dynamin and many other proteins the forming vesicle buds off from the plasma membrane (as reviewed in Pishvaee & Payne 1998).

So far four different but closely related adaptors have been identified. They are all heterotetrameric and have 2 large subunits and 2 smaller ones. The two large subunits of AP1 are called γ and $\beta 1$ and are around 100kDa, the medium subunit is called $\mu 1$ and the

small subunit σ1 and they are 47 and 19kDa in size respectively. AP1 was found to recruit clathrin to the TGN and is therefore important in budding from this compartment (as reviewed in Robinson 1994).

AP2 consist of an α and a β2 subunit which are again around 100kDa and the μ2 and σ2 subunits are 50 and 17kDa respectively. As mentioned above AP2 is important in recruiting clathrin to the plasma membrane for endocytosis (as reviewed in Robinson 1994).

AP3 consists of a δ subunit, which is 160kDa, and a β3 subunit of 120kDa. The μ3 and σ3 subunits are 47kDa and 22kDa respectively (Dell'Angelica *et al.* 1997). There is some dispute as to whether AP3 can bind clathrin, but it is thought to be important in transporting membrane proteins from the TGN to lysosomes (as reviewed in Odorizzi *et al.* 1998).

AP4 has been discovered recently and it is mainly found around the TGN. It consists of two large subunits ε (127kDa) and β4 (83kDa) and two smaller subunits μ4 and σ4, which are 50kDa and 17kDa respectively (Hirst *et al.* 1999). So far, little is known about its functional role.

Quite a different kind of adaptor protein is PACS-1 (phosphofurin acidic cluster sorting protein), which belongs to the family of the cytosolic connector proteins (Wan *et al.* 1998). This protein is probably a different kind of coat protein and connects membrane protein to the clathrin/AP1 sorting machinery at the TGN/endosomes. PACS-1 consists of a single subunit of about 120kDa (Wan *et al.* 1998).

GLUT4 is known to be endocytosed via clathrin coated pits (Slot *et al.* 1991a; Robinson *et al.* 1992) and co-localises with AP1 in intracellular vesicles near the TGN (Marsh *et al.* 1998). Indeed AP1 and AP3 have also been found on GLUT4 vesicles in rat adipocytes (Gillingham *et al.* 1999).

In this Chapter we set out to identify the adaptors present in 3T3-L1 adipocytes and determine whether GLUT4 is bound by any of these proteins under different conditions. We found that insulin had no effect on the distribution of the adaptors. Yeast two hybrid studies did not show any association between GLUT4 and the μ chain of the adaptors, but immunoprecipitation suggested a complex formation between GLUT4 and AP1 and AP3.

5.3 Results

5.3.1 Subcellular distribution of clathrin adaptors in 3T3-L1 adipocytes

In order to characterise the subcellular distribution of adaptors in 3T3-L1 adipocytes in the absence or presence of insulin, we probed a subcellular fractionation for the five different adaptors. In Figure 5.1 it can be seen that insulin does not have an effect on the distribution of any of the adaptors examined. AP1 has been reported to be found intracellularly associated with the TGN (as reviewed in Robinson 1994). In subcellular fractionations the TGN is found in the low density microsomes (LDM) (Piper *et al.* 1991; Martin *et al.* 1994) and as predicted we found the highest levels of AP1 in the

LDM fraction with lower levels also present in HDM and soluble protein fraction. AP2 on the other hand was found at the plasma membrane and this is as expected, as it is the adaptor important in clathrin coated endocytosis from the plasma membrane (as reviewed in Robinson 1994). Interestingly AP2 is the only adaptor examined here which is not found in the soluble protein fraction. Two different antibodies were used to look at the distribution of AP3. AP3 is found mostly in the LDM and soluble protein fractions with lesser amounts in the HDMs and a small amount in the PM fraction. This is as expected as AP3 has been shown by immunofluorescence to be in the perinuclear region probably located in the TGN and the endosomes (as reviewed in Odorizzi *et al.* 1998). Interestingly when using the μ chain antibody for AP3 we could detect a second band in the plasma membrane fraction and to a lesser degree in the HDMs, but not in any of the other fractions. The significance of this could not be determined. AP4, which has been identified recently (Dell'Angelica *et al.* 1999; Hirst *et al.* 1999) is found mostly in the LDM and the soluble protein fraction in 3T3-L1 adipocytes. AP4 has been shown by immunogold electron microscopy to be associated with nonclathrin-coated vesicles in the region of the TGN (Hirst *et al.* 1999) and the presence of AP4 in the LDM fraction supports this data. PACS-1 which is known to be present mainly in endosomes and the TGN (Wan *et al.* 1998) is found in the LDM fraction in 3T3-L1 adipocytes.

5.3.2 The intracellular adaptors are found in the GSV fraction on iodixanol gradients

In order to determine whether GLUT4 and the adaptors that were found in intracellular fractions are present in the same vesicle population, iodixanol gradient centrifugation was used. Figure 5.2 shows the distribution of AP1 and AP3 in LDM loaded from basal

and insulin treated cells. Both proteins were predominantly found in peak 1, particularly in the first fraction. This is surprising as Hashiramoto & James described peak 2 to contain the TGN (Hashiramoto & James 2000) and we would therefore have expected the adaptors in these fractions. The significance of this is discussed below. The results for AP4 and PACS-1 were virtually identical (results not shown). These data suggest a possible co-localisation of the intracellular adaptors and GLUT4.

5.3.3 Yeast 2 hybrid analysis of GLUT4:adaptor interactions

Adaptors are known to bind membrane proteins directly and this is important for the targeting of these proteins (Ohno *et al.* 1995; Sorkin *et al.* 1995). In order to determine whether the GLUT4 N- or C-termini could bind the μ chain of AP1-4, we used the yeast 2 hybrid system as described previously (Stephens *et al.* 1997; Stephens & Banting 1998). The yeast strain L40 was transformed with either the C-terminal or the N-terminal tail of GLUT4 fused to LexA, a DNA binding domain (vector pBTM116). The yeast was at the same time also transformed with the μ chains of the adaptors fused to VP16, a activation domain (vector pVP16) (Table 5.1). As positive controls, the previously described cytosolic domain sequences of TGN38 (transformant 26) and lgp120 (a lysosome-associated membrane protein, transformants 27 and 28) fused to the LexA gene were used as described in Table 5.1 (strength of interaction described by +) (Stephens & Banting 1998). Negative controls were the empty plasmid pVP16 against the two GLUT4 constructs (transformants 1,7,13 and 19) and against the TGN38 construct (transformant 25). Several screens were performed to look for interactions between the fusion proteins.

In the event of an interaction between the two proteins there is an activation of the two reporter genes and this can be measured either by β-galactosidase activity or by growth of the yeast on media lacking histidine. The β-galactosidase filter assays did not show any activation of transcription suggesting no interaction between either GLUT4 constructs and the μ chains of the adaptors (Figure 5.3). Similar results were obtained on plates lacking histidine and a photo of this plate is shown in Figure 5.4. Although transformant 20 seems to grow on the plate lacking histidine, which would suggest an interaction between the C-terminal tail of GLUT4 and μ1, this result could not be confirmed in a repeat of this experiment or in any of the other assays performed.

A more sensitive assay is the use of liquid cultures to determine whether there is any activity of the reporter genes. In Table 5.2 the results of the transformed yeast grown in liquid media lacking histidine are summarised. OD_{600} readings to determine yeast growth were taken after 42 and 68 hours. The positive controls (26 and 27) showed growth after this time as did transformant 15 which was confirmed in a repeat of this experiment. This would suggest an interaction of the C-terminal tail of GLUT4 with μ2. β-galactosidase activity can also be measured in a liquid culture assay using CPRG (chlorophenol red-β-D-galactopyranoside) as a substrate. β-Galactosidase can cleave CPRG and this causes a colour change from yellow to orange/red, which can be detected by measuring the OD_{578}. The results of such an assay can be seen in Table 5.3 and here the OD_{578} readings after different times are shown. The positive control 27 showed as expected a very strong interaction seen by the strong colour development after only 2 minutes. After 22 hours none of the GLUT4-construct transformants showed a clear colour development. Transformant 15 did not show any β-galactosidase activity over the background activity.

In order to test some of the transformants again, which had in different repeats of the assays given any indication of an interaction, triplicate sets of 10 transformants were used for the CPRG assay. The results with calculated β-galactosidase activity can be seen in Table 5.4. A positive interaction would be expected to have a value around 3 or more and this can only be seen in the positive controls. Importantly an interaction is only thought to be real if both reporter genes are activated. Transformant 15 is therefore unlikely to present a real interaction between GLUT4 and AP2.

Thus, by yeast two-hybrid assay we could not find any interaction between the cytosolic tails of GLUT4 and the μ chains of the adaptors.

5.3.4 Immunoprecipitations

Adaptors are thought to be recruited to the membrane receptors from the cytosol under certain circumstances. In order to mimic such an event we treated 3T3-L1 adipocytes with insulin and then "reversed" this effect either by washing the cells with a low pH buffer for different times or by adding wortmannin as described in the Figure legends. Insulin will cause a translocation of GLUT4 to the plasma membrane and both the reversal and the wortmannin treatment cause the endocytosis of GLUT4, wortmannin by increasing the rate of endocytosis (Spiro *et al.* 1996) and low pH buffer washes by removing insulin from its receptor thus prompting GLUT4 re-internalisation. Wortmannin has been shown to cause the accumulation of GLUT4 in intracellular vesicles distinct from GSVs (Malide & Cushman 1997; Chapter 3). The reversals using low pH buffer on the other hand should lead to the recruitment of GLUT4 to the GSV

compartment. These different treatments will lead to re-internalisation of GLUT4 and under these circumstances an interaction of the adaptors with GLUT4 is most likely.

In order to look for an *in vivo* interaction between the adaptor complex and GLUT4 we immunoprecipitated (IP) GLUT4 from cells treated as described above and probed these for AP1, AP2 and AP3. Due to the fact that an adaptor could bind GLUT4 either on the C-terminus or on the N-terminus, we used GLUT4 antibodies raised against the N-terminus or the C-terminus of GLUT4. It is important to note here that the N-terminal GLUT4 antibody used was whole serum, while the C-terminal GLUT4 antibody was affinity purified. In Figure 5.5 an immunoprecipitation using the C-terminal GLUT4 antibody is shown. The upper panel shows that GLUT4 was successfully precipitated, but none of the adaptors seem to interact with GLUT4. The strong band in the lower panel IP is probably due to the antibody in the preparation crossreacting with the secondary antibody used for signal-detection. Figure 5.6 shows a nearly identical experiment, but in this case the N-terminal GLUT4 antibody was used for the immunoprecipitation. Again immunoprecipitation of GLUT4 was successful seen by the absence of GLUT4 in the supernatant and by the strong band in the IP lanes. The lower part of the band in the IP fractions is thought to represent GLUT4, while the upper part again is the antibody (IgG heavy chain) present in the immunoprecipitation, which will run at about 50kDa. In this immuoprecipitation it can be seen that there is some AP1 as well as AP3 present in the IP. The presence of AP3 is confirmed by both the antibody to the large δ chain as well as to the small μ chain of the adaptor complex, although the latter is difficult to distinguish due to the crossreacting contaminating antibody. The important result in the bottom panel of Figure 5.6 is the disappearance of the lower band in the supernatant fractions and the strength of the signal in the IP fractions. This is in

strong contrast to both Figure 5.5 as well as to the negative control seen in Figure 5.7 and suggests an *in vivo* interaction of GLUT4 with AP1 and AP3.

In order to test whether IRAP, the aminopeptidase known to co-localise with GLUT4 (Ross *et al.* 1996; Martin *et al.* 1997), can also interact with the adaptors, a similar experiment was performed using an IRAP antibody for immunoprecipitation. These results are shown in Figure 5.8 and they would suggest an interaction between IRAP and AP3, but not AP1.

In conclusion this set of data suggests an *in vivo* interaction between the C-terminus of GLUT4 and AP1 and AP3 as well as an interaction between IRAP and AP3.

5.4 Discussion

GLUT4, the insulin responsive glucose transporter, has been found to be localised in clathrin lattices at the plasma membrane (Robinson *et al.* 1992) and co-localises with AP1 near the TGN (Marsh *et al.* 1998). This evidence suggests a role of the clathrin coat adaptors in the intracellular targeting of GLUT4. In this Chapter we therefore set out to characterise clathrin adaptors in the insulin sensitive 3T3-L1 adipocyte cell line. We found here that insulin did not have any effect on the subcellular distribution of the adaptors. On the one hand, this may not be surprising, as these cells were treated for 30min with 1μM insulin and this might be a too long timescale for determining the adaptor recruitment to the membrane as this might occur very quickly. Also plausible however, is that the subcellular fractionation method employed here is too crude to

detect relatively minor changes in subcellular distributions. It is of course also possible that insulin has no effect on the distribution of the adaptors. This is unlikely though as electron microscopy pictures of the plasma membrane have revealed that GLUT4 is found mainly in clathrin lattices in the absence of insulin, but in the presence of insulin GLUT4 was found both within and without clathrin lattices (Robinson *et al.* 1992). This clearly demonstrates that insulin does have an effect on the association of GLUT4 with the clathrin coat (and therefore presumably with AP2).

Adaptors are thought to be recruited to the membrane from the cytosol (as reviewed in Kirchhausen *et al.* 1997). Surprisingly our results did not show any AP2 in the cytosolic fraction, which could of course be due to the fact that samples were only taken at two different conditions (Basal and Insulin) and a presence of AP2 in the cytoplasm could therefore have been missed. However, little is known about the requirements for recruitment of AP2 to the plasma membrane and to our knowledge *in vivo* recruitment of AP2 from the cytosol to the membrane has not been shown. Binding of both AP1 and AP3 to the target membrane on the other hand is found to be regulated by the small GTPase ARF1 (as reviewed in Le Borgne & Hoflack 1998).

All of the adaptors that were found intracellularly were mainly localised to the LDM fraction. Due to the fact that this fraction contains TGN and endosomes, as well as GSVs, we tried to determine where the adaptors in this fraction were localised. Iodixanol gradients revealed all of the adaptors in the first peak corresponding to the GSV compartment. The distribution of GLUT4 and the adaptors in this peak however was quite different. While GLUT4 is usually found in fractions 2-4, the adaptors were mainly found in fraction 1 (Figure 5.2 compare with Figure 3.1A). Another observation is that

Hashiramoto & James reported peak 2 to contain the TGN (Hashiramoto & James 2000), but in our study very little of the adaptor proteins were found in this peak. One explanation for this could be that the TGN is not entirely found in peak 2 and that some is also present in the first peak. More likely though it is due to the complex formation of the adaptor proteins. Only LDM fractions are loaded on the gradient and the adaptors present in this fraction are therefore membrane associated. The presence of the adaptors in this first fraction suggests a complex formation with cytoskeletal elements as this fraction is thought to contain cytoskeleton. This method is therefore probably not accurate for determine the distribution of such proteins in the LDM fraction.

Some of the adaptors on the other hand could well be present in the GSV compartment. Gillingham *et al.* have probed GLUT4 vesicles for AP1 and AP3 and found significant amounts of both proteins on GLUT4 vesicles. Interestingly more AP1 was recruited onto the GLUT4 vesicles in the presence of GTPγS and this was partially blocked by brefeldin A suggesting an involvement of ARF in this coating process (Gillingham *et al.* 2000).

The fact that adaptors co-localise with GLUT4 in different regions of the cell led us to investigate whether there are any direct interactions between these proteins. The yeast two hybrid experiments did not show any interaction of GLUT4 with the adaptor medium chains. The absence of an interaction could be due to different methodical problems, probably in the GLUT4 constructs, as the yeast two hybrid screen using these medium chain constructs has been shown to work well with other proteins (Stephens *et al.* 1997). One such example could be the distance between the reporter gene and GLUT4 as this has been shown to be important for μ1 and μ2 binding to the tail of

TGN38 (Ohno *et al.* 1996). The lack of an interaction in our system could of course be due to the fact that these proteins do not interact *in vivo*, but this seems unlikely as the μ chains of the adaptors have been shown to recognise both Tyrosine based signals (Ohno *et al.* 1995) as well as di-leucine motifs (Rodionov & Bakke 1998) of other membrane proteins. On the other hand, although the phenylalanine at position 5 in GLUT4 is known to be important for internalisation (Piper *et al.* 1993) and is thought to be similar to a tyrosine-based motif, studies have shown that the tyrosine residue in this motif is absolutely essential for binding to μ2 and could not be substituted by a phenylalanine residue (as reviewed in Bonifacino & Dell'Angelica 1999). It is therefore not certain that this motif is involved in AP2 binding to GLUT4 and this could explain the lack of an interaction seen in the yeast 2 hybrid system.

An *in vivo* interaction between a membrane protein and an adaptor has been shown for AP2 and the epidermal growth factor receptor (Sorkin *et al.* 1996). In order to look for such an interaction between GLUT4 and the adaptors we performed immunoprecipitations of GLUT4. Our data showed an interaction of the GLUT4 C-terminus with AP1 and AP3 and an interaction of IRAP with AP3. The C-terminus of GLUT4 contains, as described earlier, a di-leucine motif as well as an acidic cluster and other sequences important for GLUT4 targeting. Rapoport *et al.* showed by using an UV-induced cross-linking reaction that the β-chain of AP1 is able to bind to the di-leucine motif of GLUT4 (Rapoport *et al.* 1998). It is therefore likely that the interaction we observe between GLUT4 and AP1 is due to β1 binding the di-leucine motif in the C-terminus of GLUT4.

AP3 has also been found to be able to bind to di-leucine motifs (Höning *et al.* 1998), but it is not known whether this interaction occurs also via the β subunit of AP3. AP1 is thought to be important in intracellular transport of membrane proteins to the endosomes, while AP3 is probably responsible for intracellular transport to lysosomes. If indeed both adaptors recognise the di-leucine motif in GLUT4 the question of specificity arises. For recognition of the Tyrosine-motif in membrane proteins by different adaptors, it is known that the residues surrounding the motif are important. It has been found that while mutation of one of the residues around the tyrosine-motif might reduce the binding to μ1 this has no effect on μ2 binding and vice versa (Ohno *et al.* 1996). This could be similar for the di-leucine motif and the surrounding sequences such as the acidic cluster might have a role in directing specificity. The di-leucine motif of GLUT4 is known to be important in targeting GLUT4 from the TGN/GSVs to the endosomes (Melvin *et al.* 1999) and the interaction between GLUT4 and AP1 observed here is therefore supported by the knowledge of a role of AP1 in this trafficking step.

IRAP contains two di-leucine motifs in its cytosolic tail (Keller *et al.* 1995), one of which has been implicated in intracellular retention of IRAP (Subtil *et al.* 2000) and the binding of AP3 found in this study could occur via these motifs. Clearly much more research has to be done to map the sites of these interactions.

In this study we could not determine an interaction between AP2 and GLUT4. This is surprising, as GLUT4 is known to be endocytosed via clathrin coated pits (Robinson *et al.* 1992) and this is thought to mediated by AP2. It could of course be possible that there is no such interaction *in vivo*. One idea to explain this could be that during endocytosis several different membrane proteins are internalised together. GLUT4 could

be recruited to a site of endocytosis in which other membrane proteins are localised and AP2 might recognise a motif in some of the proteins other than GLUT4 and endocytosis occurs taking GLUT4 along. This model would though suggest that GLUT4 could recognise the site of endocytosis, presumably by the presence of a specialised membrane receptor for GLUT4. This would reduce the specificity of targeting of GLUT4, as there is the need for another protein that internalises together under same conditions with GLUT4.

The results found here could also be explained due to problems in immunoprecipitating such a large complex. There have been studies to determine whether AP2 will first bind clathrin and then bind the membrane protein or whether it binds first to the motif of the membrane protein and then recruits clathrin to the site of endocytosis (Rapoport *et al.* 1997). If AP2 binds first to the membrane protein, an immunoprecipitation of this protein is more likely to co-precipitate AP2. If AP2 on the other hand binds clathrin first this will lead to the formation of a very big complex that might be difficult to immunoprecipitate. The lack of an immunoprecipitation of both GLUT4 and AP2 would support the formation of a complex between AP2 and clathrin first and this is supported by the fact that when clathrin and AP2 co-assemble there is an increase in affinity of the μ2 chain for the receptor tail (Rapoport *et al.* 1997). Such hypotheses could explain why other groups have failed to co-immunoprecipitate adaptor proteins with other membrane proteins (with the notable exception of the EGF receptor, Sorkin *et al.* 1996).

One other observation from our results is that the interaction between the adaptors and GLUT4 is increased in the samples treated with wortmannin and in the reversed samples. This is consistent with the role of the adaptors AP1 and AP3 in intracellular

targeting, as there should be under these conditions an increased trafficking of GLUT4 through internal membranes, dictating a need for careful sorting of cargo.

Although our immunoprecipitation results suggested an interaction between some of the adaptors and GLUT4 these results could not be consistently repeated. Due to the negative results from the yeast two hybrid studies and due to similar negative results found in other laboratories using a range of approaches (David E. James and Gwyn W. Gould personal correspondence) we did not pursue this data further.

In conclusion we found that under our experimental conditions insulin has no effect on the distribution of clathrin adaptors in 3T3-L1 adipocytes. We could not determine any interaction between the medium chains of the adaptors with the cytosolic tails of GLUT4, but *in vivo* GLUT4 seems able to bind both AP1 and AP3.

Figure 5.1 Insulin has no effect on the subcellular localisation of clathrin adaptors

3T3-L1 adipocytes were treated with or without 1μM Insulin for 30 minutes. Subcellular frationations were prepared as described in *Materials and Methods (section 2.11.1)* and the resulting membrane fractions (PM: plasma membrane, LDM: low density membrane, HDM: high density membrane and SP: soluble proteins) were separated by SDS-PAGE. 20μg of protein was loaded on each lane. Antibodies used were raised to different subunits of the adaptor complex, for AP1 against the γ subunit, for AP2 against the α subunit, for AP3 against either the δ or the μ subunit and for AP4 against the ε subunit. The position of the molecular markers are indicated on the left of the figure. Shown is a representative experiment, repeated twice with the same results.

Figure 5.1

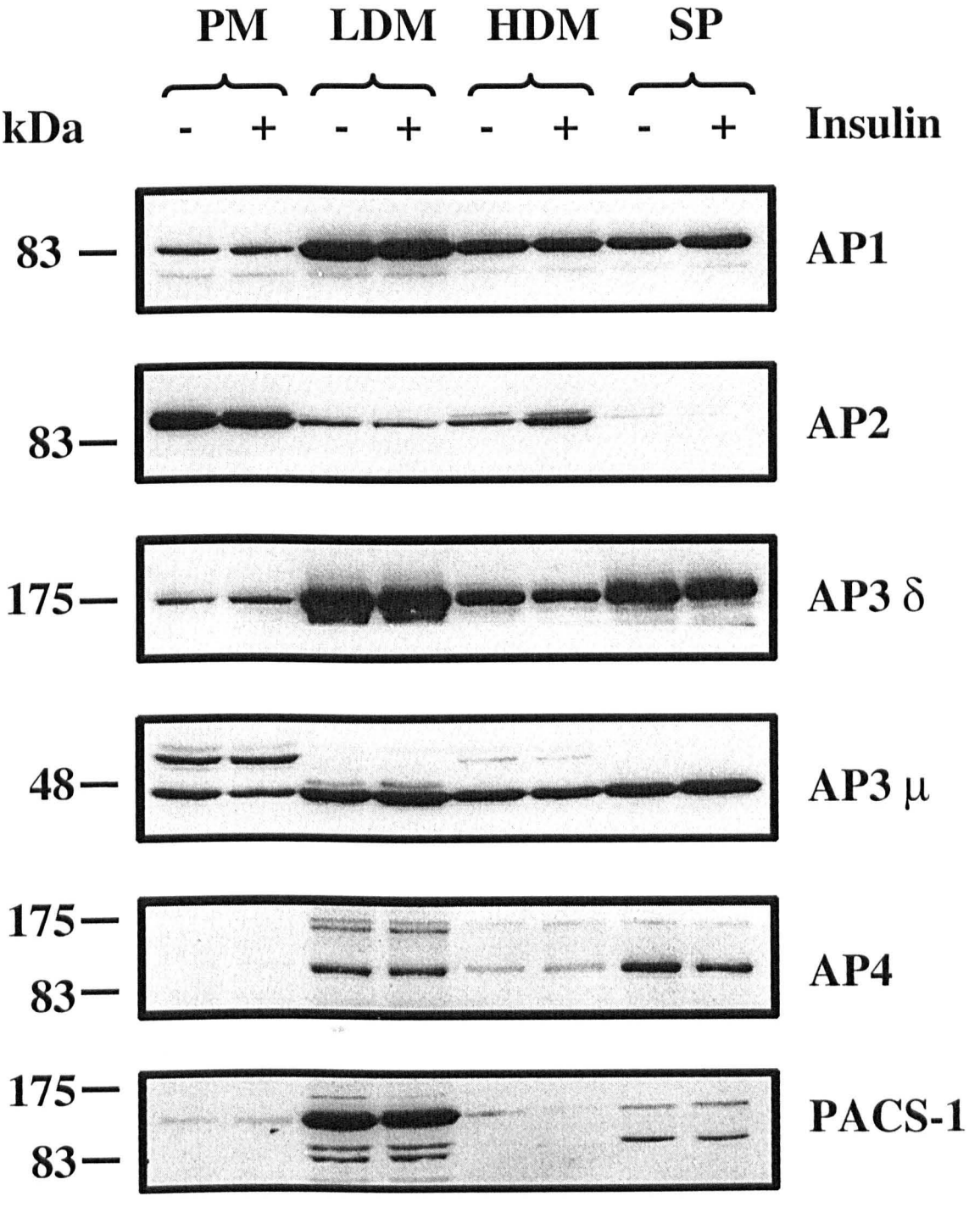

PM
LDM
HDM
SP
kDa
- + - + - + - +
Insulin
83
AP1
83
AP2
175
AP3 δ
48
AP3 μ
175
83
AP4
175
83
PACS-1

Figure 5.2 Iodixanol gradient analysis of AP1 and AP3 distribution in LDM fractions.

3T3-L1 adipocytes were treated with (I) or without (B) 1μM Insulin for 30 minutes. LDM fractions were prepared and run on an iodixanol gradient as described in *Materials and Methods (section 2.11.1 and 2.15)*. 25μl of each fraction was separated by SDS-PAGE and immunoblotted for AP1 or AP3 (δ). Shown is a representative immunoblot and the experiment was repeated with similar results.

Figure 5.2

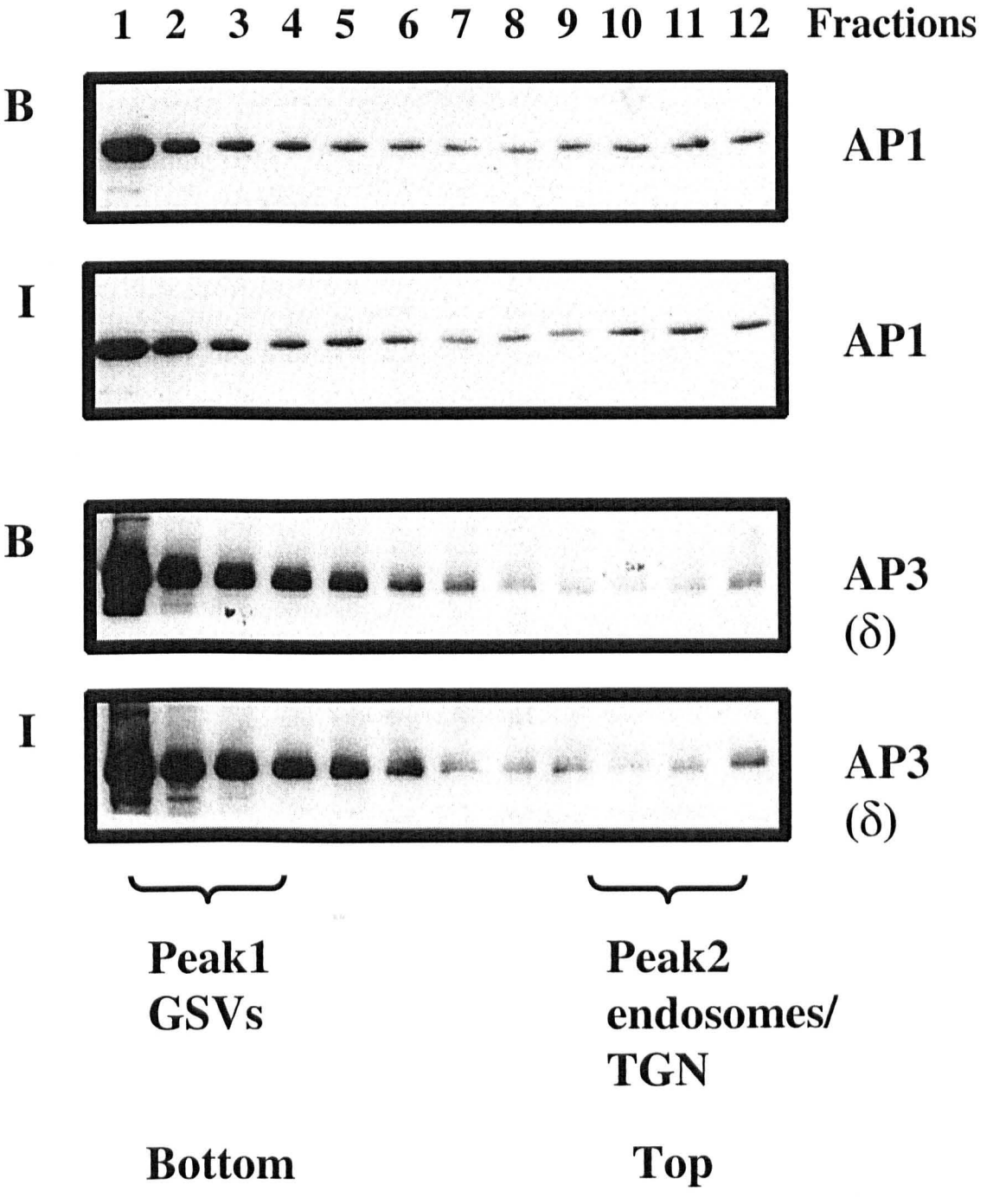

1 2 3 4 5 6 7 8 9 10 11 12 Fractions
B
AP1
I
AP1
B
AP3
(δ)
I
AP3
(δ)
Peak1
GSVs
Peak2
endosomes/
TGN
Bottom
Top

Table 5.1 28 Transformants of the yeast strain L40

N-terminal GLUT 4 construct (2 different constructs):

G4NTT contains the first 23 amino acids of the human GLUT4 cDNA. The numbers 22 and 25 represent two different constructs made exactly the same way.

	Vector containing DNA binding domain (pBTM116) fused to GLUT4		**Vector containing activation domain (pVP16) fused to adaptor μ chain**
1	G4NTT22	—	empty
2	G4NTT22	—	μ1
3	G4NTT22	—	μ2
4	G4NTT22	—	μ3A
5	G4NTT22	—	μ3B
6	G4NTT22	—	μ4
7	G4NTT25	—	empty
8	G4NTT25	—	μ1
9	G4NTT25	—	μ2
10	G4NTT25	—	μ3A
11	G4NTT25	—	μ3B
12	G4NTT25	—	μ4

C-terminal GLUT 4 construct (2 different constructs):

G4CTT encodes 42 amino acids from the region predicted to be the end of helix 12 and start of the cytosolic region onwards. The protein product of G4Tx2 is expected to be identical to G4CTT, but it also contains about 60bp of 3' untranslated region from the original GLUT4 cDNA.

	Vector containing DNA binding domain (pBTM116) fused to GLUT4/Control		**Vector containing activation domain (pVP16) fused to adaptor μ chain**
13	G4CTT	—	empty
14	G4CTT	—	μ1
15	G4CTT	—	μ2
16	G4CTT	—	μ3A
17	G4CTT	—	μ3B
18	G4CTT	—	μ4
19	G4Tx2	—	empty
20	G4Tx2	—	μ1
21	G4Tx2	—	μ2
22	G4Tx2	—	μ3A
23	G4Tx2	—	μ3B
24	G4Tx2	—	μ4

Controls

				Strength of interaction
25	TGN38-SDY	—	empty	-
26	TGN38-SDY	—	μ2	+
27	Igp120-AGYQRL	—	μ2	+++
28	Igp120-AGYQRL	—	μ3A	++

Figure 5.3 β-galactosidase filter assay to test for interaction of the fusion proteins

L40 yeast strain was transformed *Materials & Methods (section 2.17.1)* and the transformants were streaked onto selection media plates and grown until colonies had appeared. The β-galactosidase filter assay was performed as described in *Materials & Methods (section 2.17.2).* A schematic layout of the transformants on the filter is shown below. Development of blue colour indicates an interaction between the expressed fusion proteins. Shown is a picture of the filter. A similar result was obtained in a second experiment.

Order of the transformations on sqaure plates for the β-galactosidase filter assay:

1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28		

Figure 5.3

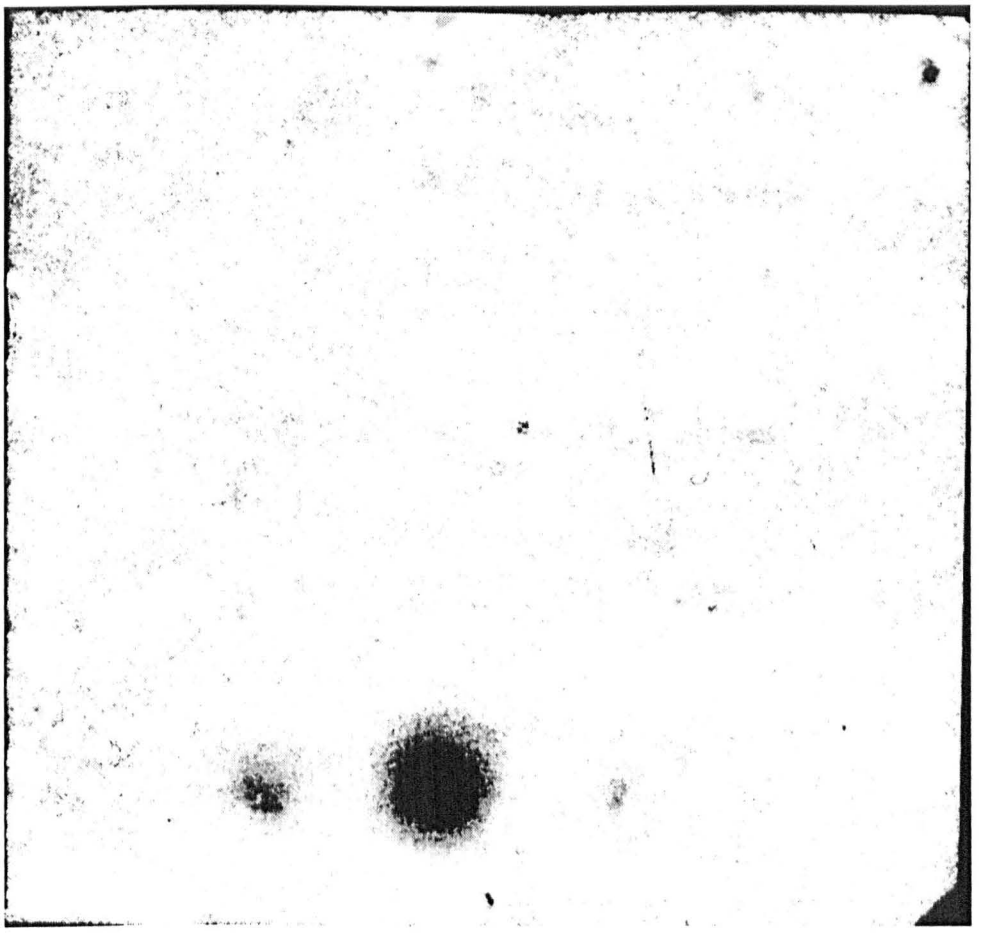


Figure 5.4 Analysis of growth of transformed yeast on media lacking histidine

L40 yeast strain was transformed as described in *Materials & Methods (section 2.17.1)* and the transformants were streaked onto selection media plates lacking histidine (section 2.16.3). A schematic layout of the transformants is shown below. Growth on media lacking histidine suggests an interaction of the expressed fusion proteins. Shown is a scan of the culture plate. A similar result was obtained in a second experiment

Order of the transformants for the minus Histidine plates

6	5	4	3	2	1
12	11	10	9	8	7
18	17	16	15	14	13
24	23	22	21	20	19
		28	27	26	25

Figure 5.4

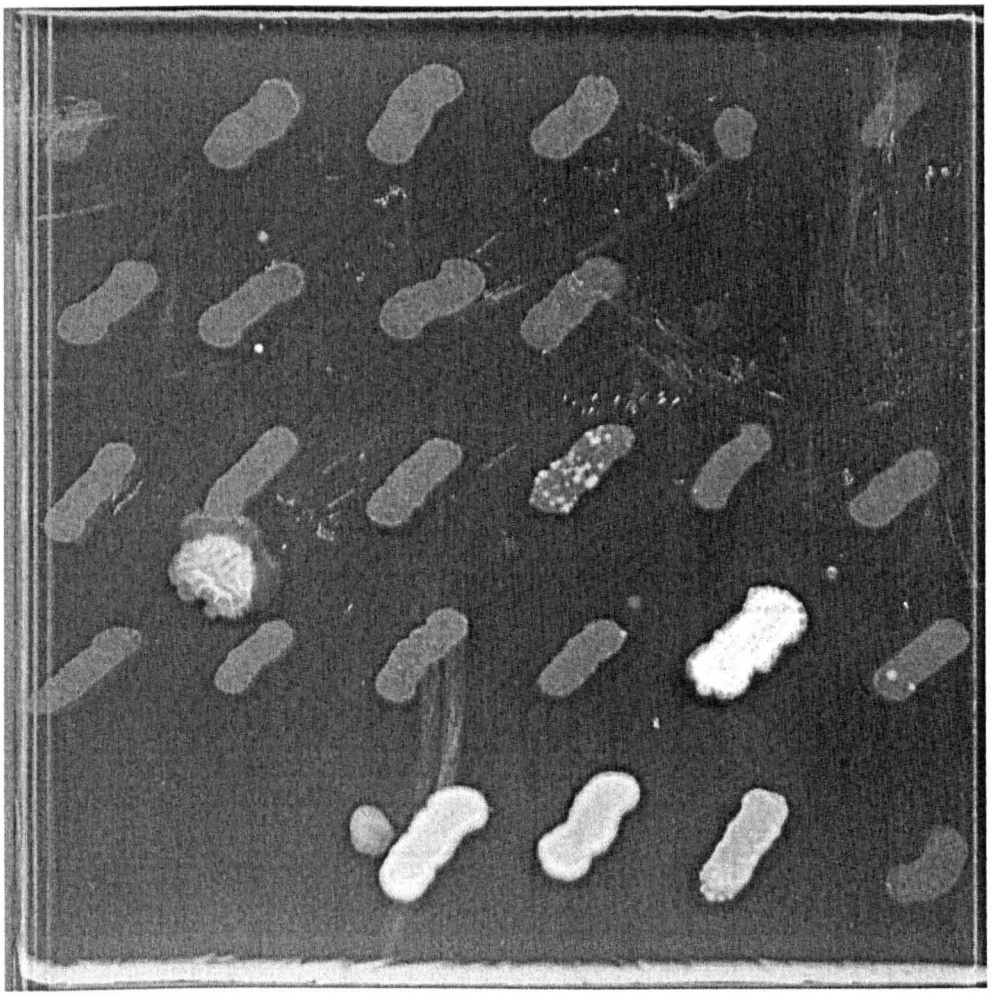


Table 5.2

Transformants	42 hours OD_{600}	68 hours OD_{600}	Transformants	42 hours OD_{600}	68 hours OD_{600}
1	0.015	0.003	15	0.052	**1.804**
2	0.015	0.002	16	0.034	0.163
3	0.014	0.001	17	0.02	0.017
4	0.016	0.001	18	0.073	0.021
5	0.009	0.023	19	0.023	0.036
6	0.012	0.01	20	0.029	0.023
7	0.014	0.015	21	0.014	0.017
8	0.003	0.013	22	0.023	0.06
9	0.013	0.012	23	0.02	0.033
10	0.003	0.021	24	0.012	0.005
11	0.005	0.01	25	0.013	0.011
12	0.013	0.015	26	0.043	**1.7**
13	0.016	0.006	27	**1.02**	**1.8**
14	0.012	0.005			

Table 5.2 Growth of transformants in Liquid minus histidine culture

Transformed yeast were grown in selection media as described in *Materials and Methods (section 2.17.4)* and OD_{600} readings were taken at indicated timepoints in order to determine growth of the yeast. Similar results were obtained in a second experiment.

Table 5.3

Transformants	OD_{578}	Stopped h/min	Transformants	OD_{578}	Stopped h/min
1	0.256	22h	15	0.37	22h
2	0.273	22h	16	0.534	22h
3	0.223	22h	17	0.459	22h
4	0.205	22h	18	0.357	22h
5	0.338	22h	19	0.336	22h
6	0.236	22h	20	0.342	22h
7	0.256	22h	21	0.285	22h
8	0.272	22h	22	0.517	22h
9	0.231	22h	23	0.333	22h
10	0.295	22h	24	0.553	22h
11	0.276	22h	25	0.218	22h
12	0.213	22h	26	**0.448**	**37min**
13	0.411	22h	27	**1.558**	**2min**
14	0.231	22h			

Table 5.3 Liquid β-galactosidase assay using CPRG as substrate

Cultures were initially grown for 4 hours without measuring the OD_{600} and the assay was performed as described in *Materials and Methods (section 2.17.5).* Colour development was stopped at the indicated times and an OD_{578} reading was taken. Due to the lack of an OD_{600} measurement at the beginning, β-galactosidase units could not be calculated.

Table 5.4

Transformants	β-galactosidase units
13	0.87
15	0.94
16	0.97
18	0.96
19	0.99
22	0.9
24	0.91
25	0.77
26	18.11
28	10.5

Table 5.4 β-galactosidase units for some transformants in liquid culture assay using CPRG as substrate

Some of the transformants were chosen for a detailed β-galactosidase assay and three cultures were set up for each. The assay was performed exactly as described in *Materials and Methods* and β-galactosidase units were calculated as described *(section 2.17.5).* The β-galactosidase units are the mean of the three separate cultures.

Figure 5.5 Immunoprecipitation of GLUT4 using an antibody against the *C*-terminus of GLUT4

3T3-L1 adipocytes were either left untreated (B), treated for 30 minutes with 1μM insulin (I), treated for 30 minutes with 1μM insulin, then washed for 5 minutes with KRMES Buffer pH 6 (section 2.2.4) (R5), treated for 30 minutes with 1μM insulin, then washed for 15 minutes with KRMES Buffer pH 6 (R15) or treated for 30 minutes with 1μM insulin, then for 15 minutes with 50nM Wortmannin (W). Immunoprecipitations were performed as described in *Materials and Methods (section 2.16),* separated by SDS-PAGE and analysed by immunoblotting. For the supernatant about 1/10th of a 10cm plate was loaded on the gel, for the immunoprecipitate about 1/3rd of a 10cm plate was loaded on the gel. The positive control is 20μg of total membrane of untreated 3T3-L1 adipocytes (no positive controls are shown for GLUT4 and AP2).

Figure 5.5

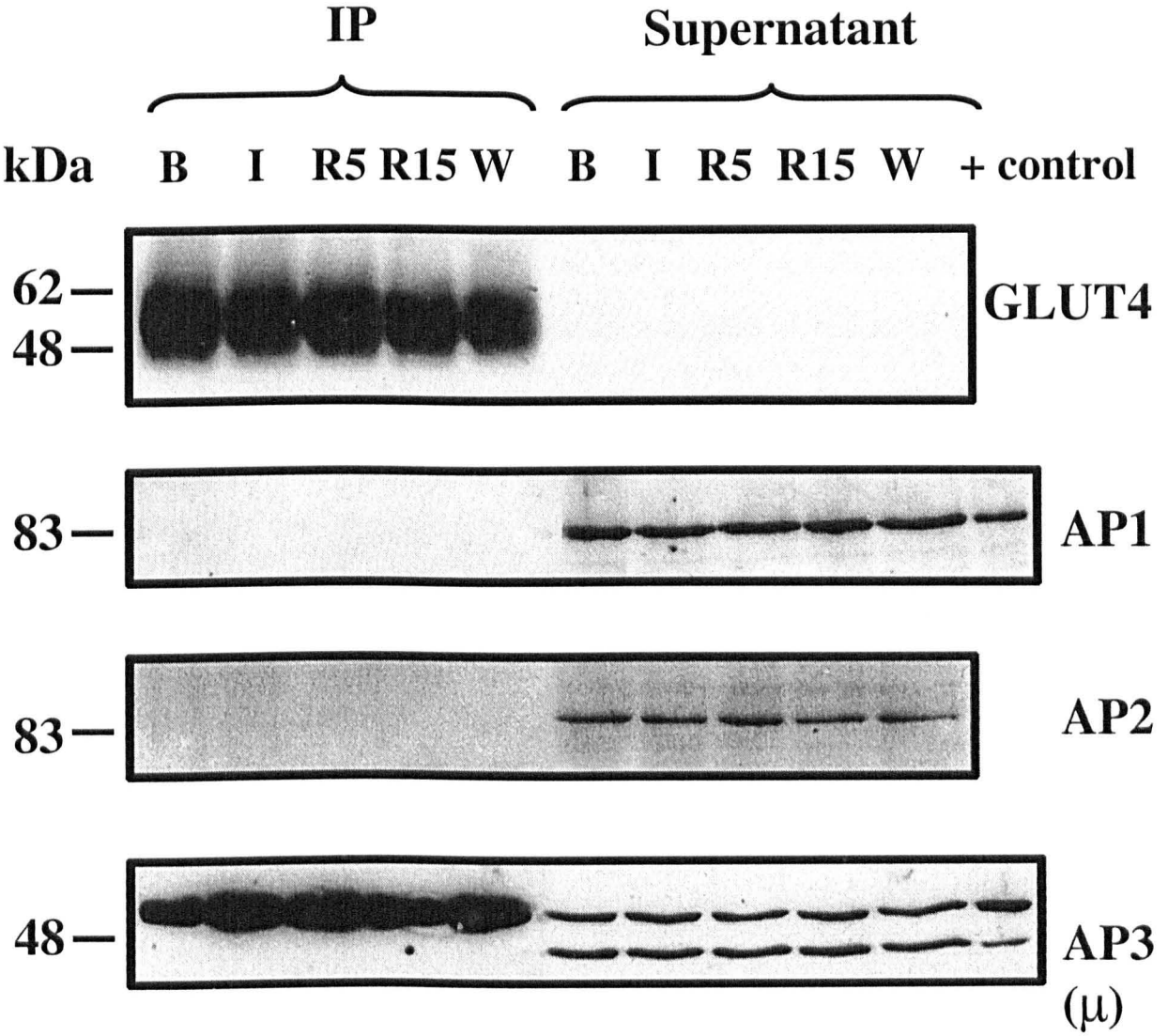

IP
Supernatant
kDa
B
I
R5
R15
W
B
I
R5
R15
W
+ control
62
48
GLUT4
83
AP1
83
AP2
48
AP3
(μ)

Figure 5.6 Immunoprecipitation of GLUT4 using an antibody against the N-terminus of GLUT4

3T3-L1 adipocytes were either left untreated (B), treated for 30 minutes with 1μM insulin (I), treated for 30 minutes with 1μM insulin, then washed for 5 minutes with KRMES Buffer pH 6 (section 2.2.4) (R5), treated for 30 minutes with 1μM insulin, then washed for 15 minutes with KRMES Buffer pH 6 (R15) or treated for 30 minutes with 1μM insulin, then for 15 minutes with 50nM Wortmannin (W). Immunoprecipitations were performed as described in *Materials and Methods (section 2.16),* separated by SDS-PAGE, and analysed by immunoblotting using the antibodies indicated. For the supernatant about 1/10th of a 10cm plate was loaded on the gel, for the immunoprecipitate about 1/3rd of a 10cm plate was loaded on the gel. The positive control is 20μg of total membrane of untreated 3T3-L1 adipocytes (no positive control is shown for GLUT4).

Figure 5.6

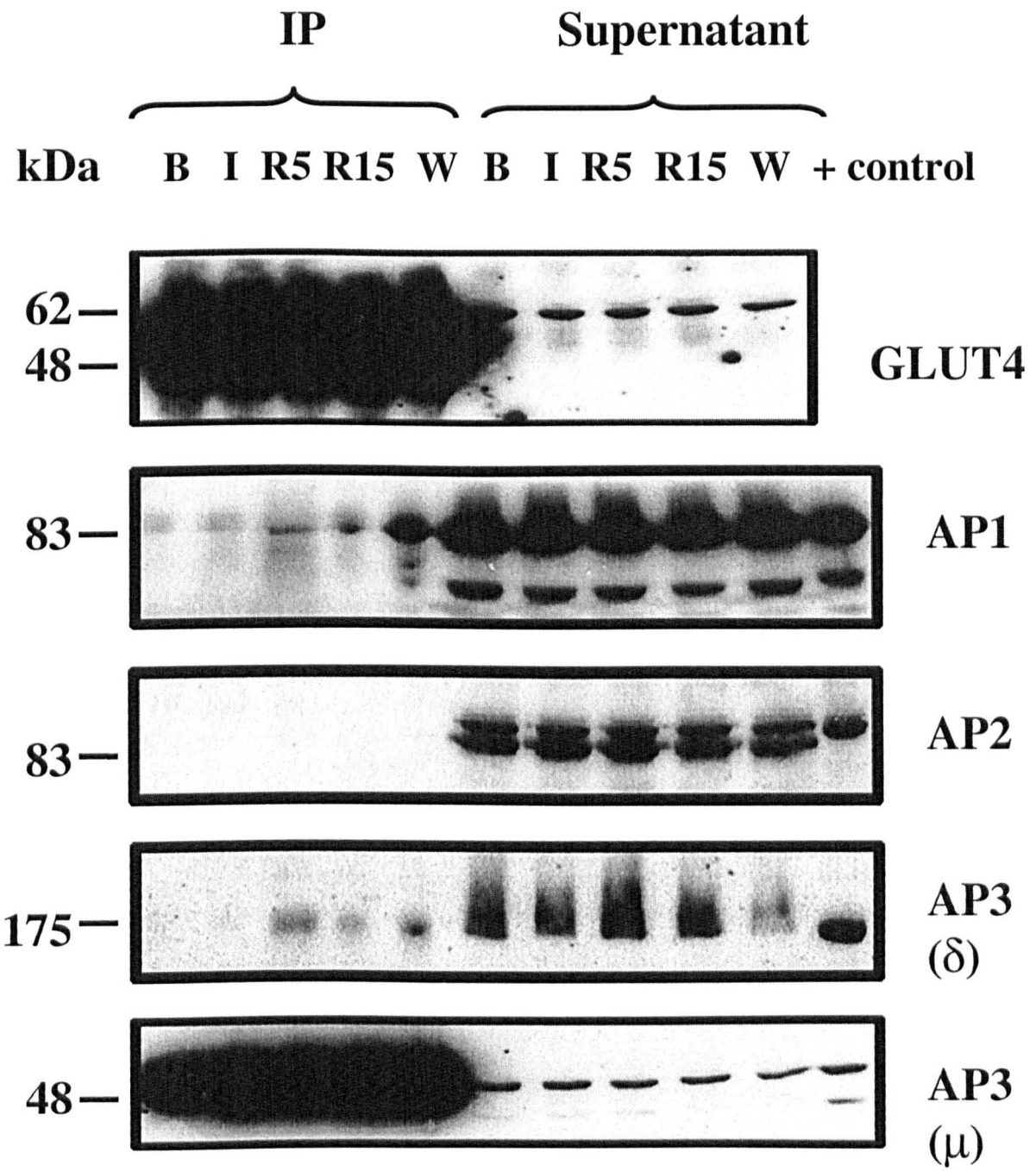

IP
Supernatant
kDa
B I R5 R15 W B I R5 R15 W + control
62
48
GLUT4
83
AP1
83
AP2
175
AP3
(δ)
48
AP3
(μ)

Figure 5.7 Immunoprecipitation using a random antibody as control for non-specific binding

3T3-L1 adipocytes were either left untreated (B), treated for 30 minutes with 1μM insulin (I), treated for 30 minutes with 1μM insulin, then washed for 5 minutes with KRMES Buffer pH 6 (section 2.2.4) (R5), treated for 30 minutes with 1μM insulin, then washed for 15 minutes with KRMES Buffer pH 6 (R15) or treated for 30 minutes with 1μM insulin, then for 15 minutes with 50nM Wortmannin (W). Immunoprecipitations were performed as described in *Materials and Methods (section 2.16),* separated by SDS-PAGE and analysed by immunoblotting using the antibodies indicated. For the supernatant about 1/10th of a 10cm plate was loaded on the gel, for the immunoprecipitate about 1/3rd of a 10cm plate was loaded on the gel. The positive control is 20μg of total membrane of untreated 3T3-L1 adipocytes (no positive control is shown for GLUT4).

Figure 5.7

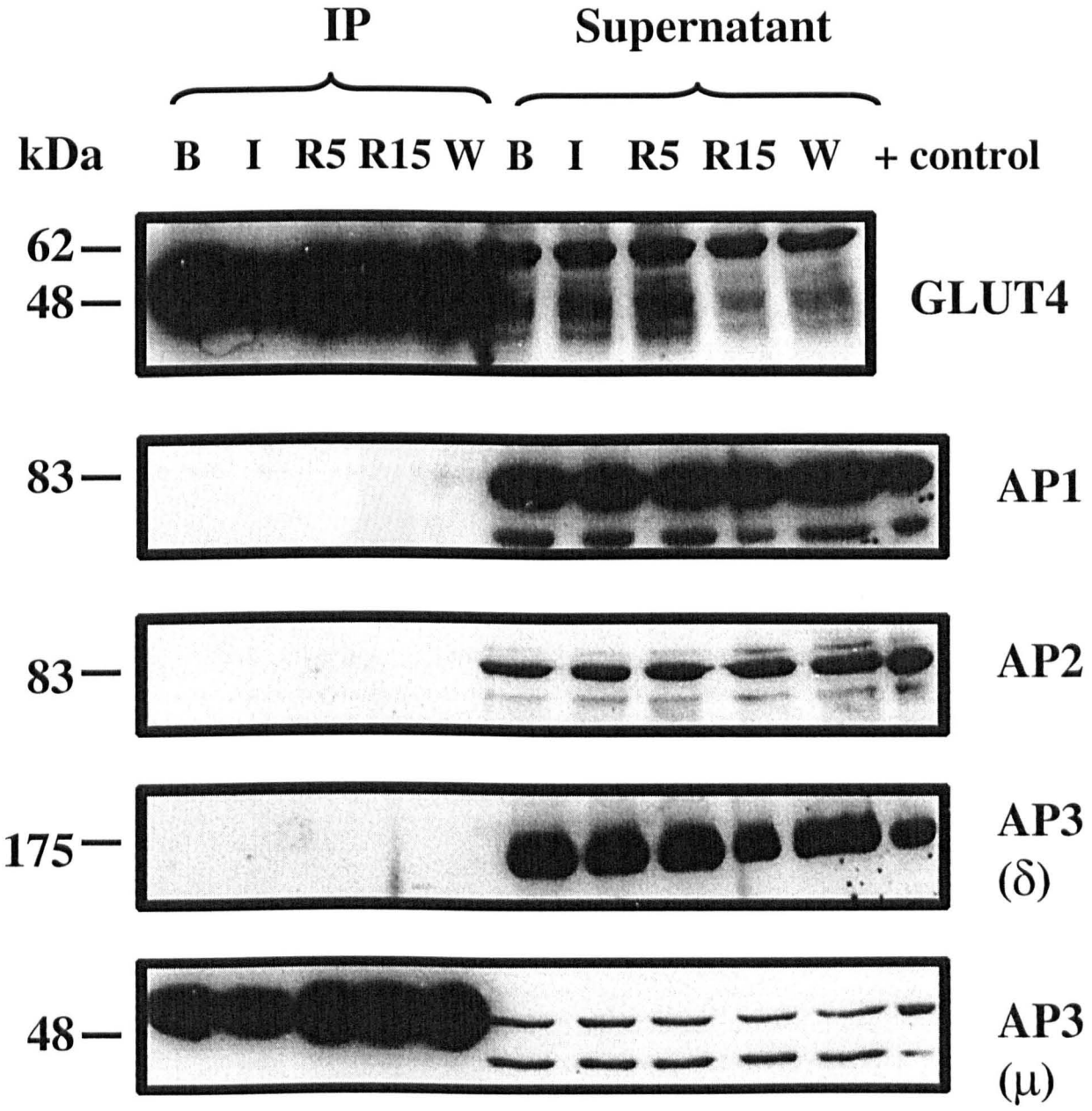
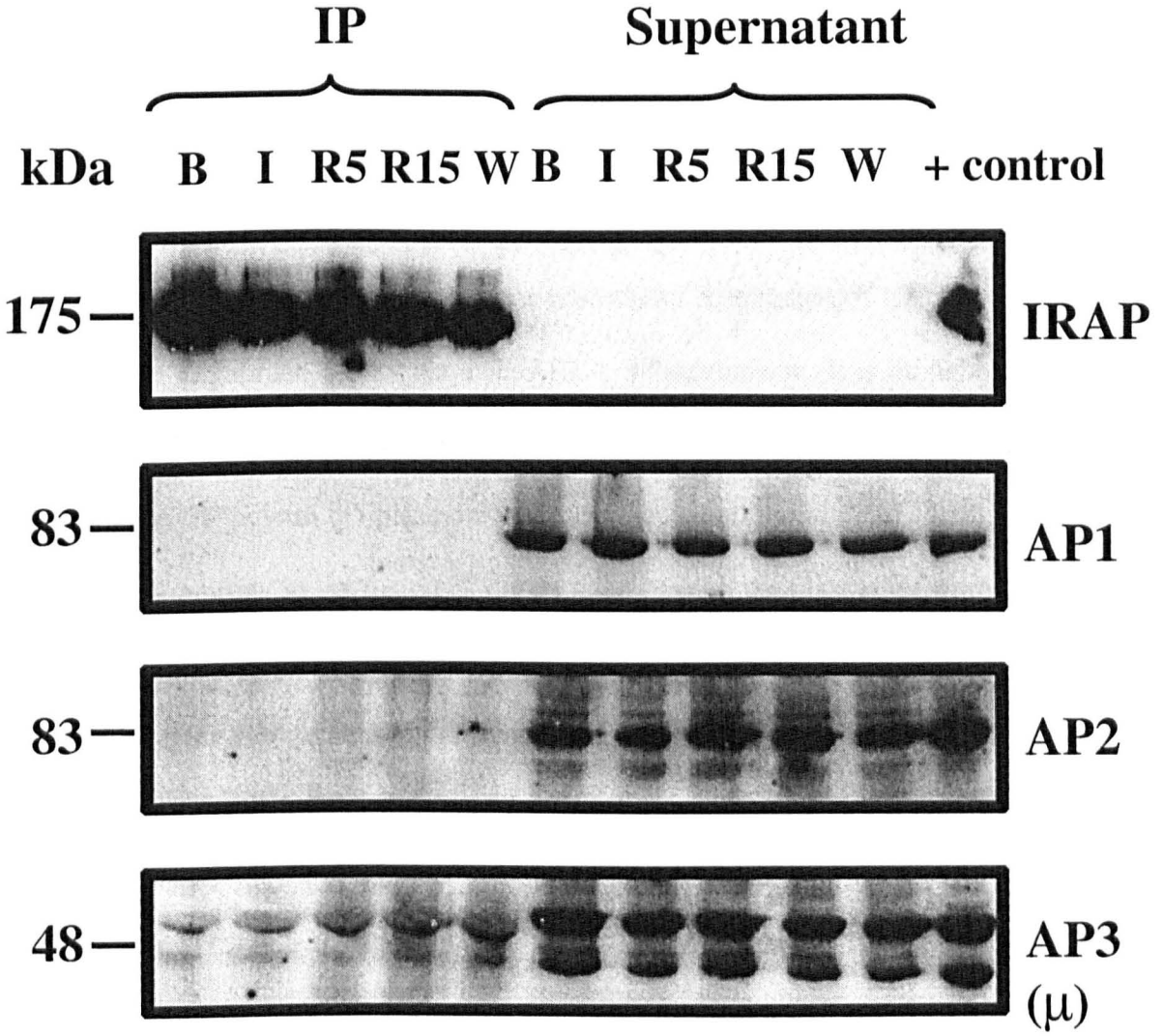

IP
Supernatant
kDa
B I R5 R15 W B I R5 R15 W + control
62
48
GLUT4
83
AP1
83
AP2
175
AP3
(δ)
48
AP3
(μ)

Figure 5.8 A possible co-immunoprecipitation of IRAP with AP3

3T3-L1 adipocytes were either left untreated (B), treated for 30 minutes with 1μM insulin (I), treated for 30 minutes with 1μM insulin, then washed for 5 minutes with KRMES Buffer pH 6 (section 2.2.4) (R5), treated for 30 minutes with 1μM insulin, then washed for 15 minutes with KRMES Buffer pH 6 (R15) or treated for 30 minutes with 1μM insulin, then for 15 minutes with 50nM Wortmannin (W). Immunoprecipitations were performed as described in *Materials and Methods (section 2.16))*, separated by SDS-PAGE and analysed by immunoblotting using the antibodies indicated. For the supernatant about 1/10th of a 10cm plate was loaded on the gel, for the immunoprecipitate about 1/3rd of a 10cm plate was loaded on the gel. The positive control is 20μg of total membrane of untreated 3T3-L1 adipocytes.

Figure 5.8


IP
Supernatant
kDa
B
I
R5
R15
W
B
I
R5
R15
W
+ control
175
IRAP
83
AP1
83
AP2
48
AP3
(μ)

CHAPTER 6 - OVERVIEW

Insulin stimulates the uptake of glucose into storage tissues such as muscle and fat, and this is mediated by the insulin responsive glucose transporter GLUT4. In the absence of insulin GLUT4 is intracellularly sequestered and it is now well established that GLUT4 occupies at least two different intracellular locations, one being of endosomal character and the other is thought to be a highly insulin responsive compartment, which is specific to GLUT4 and some other proteins such as IRAP. Insulin is known to cause the translocation of these GLUT4 storage vesicles (GSVs) to the plasma membrane and the subsequent insertion of GLUT4 in the plasma membrane. The fusion of GLUT4 vesicles with the plasma membrane occurs via a SNARE-type mechanism. It is thought that the v-SNARE VAMP2 together with the t-SNAREs syntaxin4 and syndet are responsible for fusion of the GSVs with the plasma membrane, while cellubrevin and corresponding t-SNAREs are important in the fusion of the endosomes with the plasma membrane.

A new method for the separation of the two intracellular GLUT4 pools has been described recently (Hashiramoto & James 2000), and in Chapter 3 we confirmed the validity of this method for studies in 3T3-L1 adipocytes and human adipocytes. Iodixanol gradient centrifugation analysis supports the hypothesis that the GSVs are indeed far more insulin sensitive than the endosomes and that VAMP2 is the SNARE protein responsible for GSV translocation while cellubrevin is found mainly localised to endosomes. In this Chapter we also looked at the differentiation of 3T3-L1 fibroblasts into adipocytes in order to determine at what time during differentiation the GSV compartment forms. Our data, using compartment ablation and iodixanol gradient

centrifugation, suggests that the GSVs form at about the same time that GLUT4 starts to get expressed. This confirms the idea that the insulin responsive compartment is defined by the presence of GLUT4, and IRAP gets sorted into this compartment during differentiation only once GLUT4 is present. Current studies are under way to look whether a tagged GLUT4 expressed in fibroblasts and during differentiation can drive the formation of the GSV or whether the expression of endogenous GLUT4 is needed for GLUT4 compartment formation.

Type 2 diabetes mellitus is a rapidly growing disease in the Western world and is known to be due to a defect in the translocation of GLUT4 to the plasma membrane. Although there are a large number of sites that could lead to this defect, it is now thought that it might be due to either a defective GLUT4 translocation mechanism or a mis-targeting of GLUT4. In Chapter 4 we examined insulin resistant 3T3-L1 adipocytes and looked for cellular changes which might lead to this defect. We found that SNARE proteins, and particularly the SNAREs involved in the translocation of the GSVs to the plasma membrane, are upregulated. Although this might be an adaptive rather then a causal effect, we suggest that normal SNARE protein levels are important for their correct function. We further found that GLUT4 gets depleted specifically from the GSV compartment in insulin resistant 3T3-L1 adipocytes and that this can explain the decreased insulin sensitivity of those cells. This data also suggests a block in the sorting step from the endosomes to the GSVs in insulin resistant cells, due to the fact that IRAP now largely co-localised with the transferrin receptor. In order to further look into this effect it would be of great interest to examine insulin resistant muscle as this could explain whether there is indeed a defect in the GLUT4 sorting to the GSVs and whether GLUT4 is mis-targeted to a non-insulin responsive compartment.

Many membrane proteins constantly recycle between intracellular compartments and the plasma membrane and the targeting of these proteins to their correct location is therefore crucial for the functioning of the cell. It is now thought that clathrin adaptors are important in the targeting of the transferrin receptor, TGN38 and many other membrane proteins and this led us to examine these adaptors in some detail in Chapter 5. Although insulin did not have any effect on the gross distribution of the adaptors in 3T3-L1 adipocytes, subtle effects in adaptor localisation may have been missed. It would be interesting to examine the distribution of the adaptors at different time-points after insulin administration using more sensitive methods, such as immuno- electron microscopy. The lack of an *in vitro* interaction between GLUT4 and the μ subunit of the adaptors may be explained a) by the fact that the F^5QQI^8 has not actually been shown to bind the μ chain of the adaptors although it is supposed to resemble the tyrosine-based motif and b) by the observation that the di-leucine motif is thought to bind the β subunits of the adaptors rather then the μ subunits. *In vivo* on the other hand we found an interaction between GLUT4 and AP1 and AP3, as well as IRAP and AP3, but these results could not be repeated and therefore more work is required to confirm this observation. An interaction of a membrane protein with the adaptors, although expected, has only been shown for very few proteins. If the interaction between GLUT4 and AP1 and AP3 is real this could provide more information on GLUT4 trafficking. A defect in this interaction could lead to mis-targeting of GLUT4 and this might be of great interest in the search for the cause of insulin resistance.

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