THE SUBCELLULAR DISTRIBUTION AND INSULIN-RESPONSIVENESS OF FACILITATIVE GLUCOSE TRANSPORTERS IN TRANSFECTED CELLS

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ABSTRACT

Specific anti-peptide antibodies, raised against the C-terminal portion of mouse GLUT 3 were used to examine the tissue distribution of GLUT 3 protein in various mouse tissues. Previous studies have shown that in human tissues GLUT 3 mRNA is detectable in a wide range of tissues but that GLUT 3 protein levels are highest in the brain, with lower levels being detectable in heart, placenta and fat. Results from this investigation indicated that, in contrast to humans, the expression of GLUT 3 protein in mice was restricted to the brain and neurally derived cells only.

An investigation to determine the levels of GLUT 3 protein within different rat brain regions was then carried out since *in situ* hybridisation analysis of GLUT 3 in the mouse brain had previously indicated that the expression of this isoform was not homogeneous throughout all regions of the brain. After demonstrating that anti-GLUT 3 antibodies were able to cross-react with rat GLUT 3 protein, an examination of GLUT 3 protein expression levels in individually dissected rat brain regions was carried out. The levels of GLUT 1 protein were also determined since this transporter constitutes the blood-brain barrier transport protein. Results indicated that when GLUT 1 and GLUT 3 levels were expressed relative to the levels measured in the frontal cortex, the trend was such that GLUT 1 levels increased, while GLUT 3 levels decreased towards the more posterior brain regions. It was therefore postulated that a low level of one transporter is compensated for by the expression of another. In addition, rat brain regions were screened for the expression of GLUT 2 and GLUT 4 protein. GLUT 4 expression was detected in three regions;- the pituitary, hypothalamus, and cerebellum while GLUT 2 was detected in all brain regions analysed. The detection of these latter proteins may indicate that brain regions possess a "glucose-sensing" mechanism similar to the GLUT 2-expressing β-cells of the pancreas, and that particular regions may be insulin-sensitive.

GLUT 2 and GLUT 4 are expressed in insulin-responsive tissues, liver and fat respectively, yet only GLUT 4 undergoes translocation from an intracellular region to the plasma membrane following insulin stimulation. In an attempt to determine whether translocation is a process governed by the type of cell in which the glucose transporter protein is expressed or whether it is a property encoded in the protein sequence, GLUT 2 was expressed in an adipocyte cell environment (3T3-L1 cells) and GLUT 4 was expressed in a liver-type cell environment (H4IIe cells). Immunoblotting of cell lysates prepared from GLUT 2-transfected 3T3-L1 cells and GLUT 4-transfected H4IIe cells with anti-GLUT 2 and anti-GLUT 4 antibodies revealed that GLUT 4 and GLUT 2 proteins were expressed in the relevant cells. Results indicated that GLUT 4-expressing H4IIe cells exhibited a greater increase in

2-deoxyglucose uptake in response to insulin relative to non GLUT 4-expressing cells. However GLUT 4 translocation in response to insulin could not be conclusively demonstrated. GLUT 2-expressing 3T3-L1 adipocytes exhibited a significant increase in basal 2-deoxyglucose uptake relative to non GLUT 2-expressing cells, but an approximate 3 to 6 -fold reduction in insulin-stimulated 2-deoxyglucose uptake. GLUT 2 protein was predominantly localised to the plasma membrane in both basal and insulin-stimulated cells, and did not exhibit significant translocation in response to insulin. This reduced insulin-stimulated glucose transport could not be explained by a down-regulation in GLUT 4 expression in these GLUT 2-expressing cells since GLUT 4 levels were similar in both GLUT 2 and non-GLUT 2 expressing adipocytes. The nature of the reduced sensitivity to insulin with regard to glucose transport in GLUT 2-expressing adipocytes thus remains to be elucidated.

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"The only way to atone for being occasionally a little over-dressed is by being always absolutely over-educated"

Oscar Wilde

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ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DeGlc	2-Deoxy-D-glucose
DFP	Diisopropylfluorophosphate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid, disodium salt
Ep 64	Trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane
FITC	Fluorecein isothiocyanate
GLUT	Glucose transporter
HEPES	N-2-hydroxyethylpiperazine-N' 2-ethane sulphonic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin gamma
KRP	Krebs ringer phosphate
mRNA	messenger ribonucleic acid
O.D.	Optical density
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylenediamine
Tris	Tris(hydroxymethyl)aminoethane
U.V.	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Sugar transport across biological membranes

The transport of sugar into a cell is the first and perhaps most important step in sugar metabolism. Regulation of sugar entry is a common biological strategy for the modulation of, and response to glycolytic flux and cellular activities.

The passage of glucose across cell membranes can occur in one of three ways; simple, non carrier mediated diffusion, facilitated diffusion and active transport. Simple diffusion through the lipid bilayer of the membrane only becomes significant when the diffusing substrate is lipid soluble.

There are specific proteins within biological membranes which are capable of mediating the passage of glucose and to date two functionally distinct carriers, passive and active, have been identified. Active transport systems require energy to drive the uptake of solute against its concentration gradient. The driving force for the uptake is usually derived from an electrochemical gradient of sodium ions or protons across the membrane. Active sugar transporters are found in many bacterial and yeast organisms which rely on sugar present in the extracellular environment for energy. Hence these organisms require uptake systems which are highly efficient to extract the maximum amount of sugar possible. Active sugar transporters do however exist in specific mammalian tissues such as the renal proximal brush border membrane (Turner and Moran, 1982) and the apical membrane of the intestinal mucosa (Hopfer, 1987) where there is a requirement for transepithelial sugar transport against its gradient. In mammalian cells active glucose transport is mediated by the Na+/glucose co-transporter (Maenz and Cheeseman, 1987) which, as the name suggests, mediates the entry of both glucose and Na^+ ions into the cell. Na^+ ions entering the cell are

then pumped out again by a Na^+/K^+ ATPase which creates the Na^+ gradient driving glucose uptake.

Passive glucose transport can occur through protein pores or through specific facilitative diffusion proteins. Pore proteins exhibit little substrate specificity and allow most molecules, which are small enough in size and not restricted by charge, to pass through the pore and hence cross the lipid bilayer. These pore proteins however only exist within intracellular membranes, such as the outer mitochondrial membrane, and do not exist within the plasma membrane.

Facilitative D-glucose transporter proteins have been found in most, if not all, mammalian cells studied to date. These transporters are specific for the D-enantiomer of glucose and mediate the transport of this sugar down its chemical gradient. It has been discovered that different mammalian cell types express functionally distinct facilitative D-glucose transporters which relate to their specific function and role in whole body glucose homeostasis.

1.2.1 Mammalian facilitative D-glucose transporters

A family of facilitative glucose transporters has been discovered in mammalian cells. To date the glucose transporter (GLUT) family has 7 members which have been designated GLUTs 1-7 on the basis of the chronological order of the identification of their cDNAs (for review see Bell *et al.*, 1990; Baldwin, 1993; Gould and Holman, 1993).

The first transporter to be identified was the erythrocyte transporter, GLUT 1, which was subsequently cloned and sequenced by Mueckler *et al.* (1985). Other glucose transporter isoforms were then identified in other mammalian tissues, generally through the screening of cDNA libraries at low stringency with GLUT 1 cDNA probes.

Although the facilitative transporter family encompasses 7 family members, only 6 of these (GLUTs 1-5 and GLUT 7), are functional transporters. GLUT 6,

identified by Kayano *et al.* through the homology screening approach, was found to be a pseudogene-like sequence (Kayano *et al.*, 1990). The characteristics of the individual glucose transporter isoforms are shown in Table 1.1 and are detailed more fully in the following sections.

The sizes of the mammalian glucose transporters vary from 492 to 524 amino acids but all six sequenced sugar transporters (GLUT 1, GLUT 2, GLUT 3, GLUT 4, GLUT 5, GLUT 7) have over 25% identity between their aligned amino acid residues (Bell *et al.*, 1990).

Using hydropathy plot data and amino acid sequence data, to predict secondary structure, Mueckler *et al.* proposed a model for the three dimensional orientation of the GLUT 1 protein within the plasma membrane (Mueckler *et al.*, 1990). Analyses subsequently performed on other members of the transporter family suggested that their arrangement within the lipid bilayer would be similar to that of GLUT 1. The proposed structural model predicts that the glucose transporter proteins comprise 12 α-helical membrane spanning regions with the NH_2 and COOH -termini both located cytoplasmically (see Fig 1.1). The length and sequence of these termini however varies considerably between the different transporter isoforms. A large intracellular loop exists between transmembrane regions 6 and 7 which divides the transporter structure into 2 halves, the N-terminal half and the C-terminal half. There also exists a large extracellular loop between transmembrane helices 1 and 2 which is variable in size between transporter isoforms and has potential sites for asparagine-linked glycosylation. Short segments of between 7 and 14 amino acids connect the remaining transmembrane spanning regions. The variability in length and sequence of the intracellular and extracellular loops between isoforms suggests that these non-conserved regions may have a role to play in the differential regulation and kinetics of the individual transporters.

The expression of different glucose transporter isoforms in mammalian cells is tissue specific and it is this pattern of expression which probably reflects, not only

TABLE 1.1 Mammalian facilitative D-glucose transport proteins

Isoform	Size (in amino acids)	Major sites of expression	References
GLUT 1	492	erythrocyte; placenta; brain; blood-tissue barriers; adipose and muscle; tissue culture cells; transformed cells	1 2
GLUT 2	524	Liver; pancreatic β–cells; kidney proximal tubule; small intestine	3 4
GLUT 3	496	Brain; nerve cells; small intestine	5
GLUT 4	509	skeletal muscle; heart; brown and white adipocytes	6, 7, 8, 9,10.
GLUT 5	501	Small intestine (apical membranes); testis; brain; muscle; adipose tissue (low levels)	11
GLUT 7	528	endoplasmic reticulum of hepatocytes	12

References

1. Mueckler *et al.*, (1985); 2. Birnbaum *et al.*, (1986;) 3. Fukomoto *et al.*, (1988); 4. Thorens *et al.*, (1988); 5. Kayano *et al.*, (1988); 6. Fukomoto *et al.*, (1989); 7. James et *al.*, (1989); 8. Birnbaum (1989); 9. Charron *et al.*, (1989); 10. Kaestner *et al.*, (1989); 11. Kayano *et al.*, (1990); 12. Waddell *et al.*, (1992).

the tissues own particular requirement for glucose, but also its role within whole body glucose homeostasis.

1.2.2 GLUT 1 -the erythrocyte-type glucose transporter

GLUT 1, sometimes referred to as the human erythrocyte-type facilitative D-glucose transporter, is possibly the most well studied and best characterised of the isoforms of the glucose transporter family.

Human red blood cells provide a rich source of GLUT 1 protein with the transporter comprising greater than 5% of the total erythrocyte ghost protein (Allard and Lienhard, 1985). Consequently this is the only isoform, as yet, to have been purified to near homogeneity in functional form (Baldwin *et al.*, 1982).

The purification of GLUT 1 protein and the subsequent generation of antibodies to this isoform led to the isolation of clones encoding the transporter. Mueckler *et al.*, screening an expression cDNA library prepared from the human HepG2 hepatoblastoma cell line, identified a cDNA clone encoding a 492 amino acid protein which was demonstrated to be identical to that of the erythrocyte transporter (Mueckler *et al.*, 1985). A short time later, Birnbaum *et al.* identified and sequenced an equivalent cDNA clone coding for a protein of 492 amino acids from an adult rat brain cDNA library (Birnbaum *et al.*, 1986).

GLUT 1 is an integral membrane protein which resolves on SDS-PAGE with a molecular weight of 45,000-55,000 daltons due to a single heterogeneous asparagine-linked oligosaccharide (Gorga *et al.*, 1979).

This transporter is the most widely expressed of all the transporter isoforms and its 2.8 kb mRNA has been detected in most mammalian tissues. GLUT 1 is the predominant facilitative glucose transporter isoform expressed in cultured cells and indeed in some cell lines it is the only glucose transporter protein expressed (Flier *et al.*, 1987). The protein is most abundant in the human erythrocyte, in foetal tissues, in the brain, and at blood-tissue barriers, such as the blood-brain barrier (Boado and

FIGURE 1.1

PROPOSED TOPOLOGY OF THE D-GLUCOSE TRANSPORT PROTEIN WITHIN THE LIPID BILAYER

Analyses of the predicted amino acid sequences of the mammalian glucose transporters have indicated that they are highly homologous with one another. The predicted structure for the mammalian D-facilitative glucose transporter protein (shown opposite) ecompasses 12 α–helical membrane-spanning regions with the NH_2 and COOH -termini intracellularly localised. There are large loops between helices 1 and 2 and helices 6 and 7. N-linked glycosylation can occur in the extracellular loop between helices 1 and 2 as shown. Conserved amino acids are indicated by the appropriate single-letter code and filled circles indicate conserved substitutions.

(reproduced from Gould and Holman, 1993)

in
out
N
C
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII

Pardridge, 1990), the blood-nerve barrier (Froehner *et al.*, 1988), and the blood-eye barrier of the optic nerve (Harik *et al.*, 1990).

Its ubiquitous expression, together with its high affinity for D-glucose (K_m 1-2mM) (Wheeler and Hinkle, 1985) probably reflects it role as a "house-keeping" glucose transporter, functioning to supply the cells' basic glucose requirements. Hence this transporter in most tissues is most likely to be responsible for constitutive glucose uptake.

1.2.3 GLUT 2-the liver-type glucose transporter

The second facilitative D-glucose transporter to be cloned was GLUT 2, sometimes termed the liver-type transporter.

Studies by Goresky (1967) and Williams *et al.*,(1968) provided evidence for the existence a carrier-mediated glucose transport system in liver. However, based on differences in binding constants of cytochalasin B, a potent inhibitor of GLUT 1, and on the apparent differences in the K_m for glucose, several investigators concluded that the glucose transport system in liver cells could not be accounted for by the erythrocyte-type facilitative glucose transporter GLUT 1 (Axelrod and Pilch, 1983; Maenz and Cheeseman, 1987). Furthermore GLUT 1 protein could not be detected in liver (Flier *et al.*, 1987; Birnbaum *et al.*, 1986).

Using a cDNA encoding the rat brain glucose transporter GLUT 1, Thorens *et al.* (1988) isolated a clone, from a rat liver cDNA library, encoding a 522 amino acid protein which was 55% identical in sequence to the rat brain transporter. At the same time Fukomoto *et al.* (1988) independently isolated a cDNA clone from an adult human liver cDNA library. This clone encoded a 524 amino acid protein which was 55.5% identical in amino acid sequence to the erythrocyte transporter GLUT 1. This transporter protein, designated GLUT 2, was subsequently found to be expressed in the sinusoidal membranes of hepatocytes, in the kidney, in serosal membranes of the small intestine (Thorens *et al.* 1990) and in the β–cells of the

pancreas (Birnbaum *et al.*, 1986; Orci *et al.*, 1989). Western blotting studies have revealed however that the apparent molecular mass of the protein differs between tissues, the calculated molecular weights being 53 kDa in the liver, 61 kDa in the intestine, 57 kDa in the kidney and 55 kDa in islet β-cells (Orci *et al.*, 1989; Thorens *et al.*, 1988). This variation in the size of GLUT 2 most likely reflects differences in the extent of glycosylation.

Although there are substantial amino acid sequence differences between GLUTs 1 and 2 and they both probably adopt a similar 3 dimensional structure within the plasma membrane since hydropathy plot patterns for both proteins have been shown to be similar (Thorens *et al.*, 1988). The longer extracellular loop of GLUT 2, located between transmembrane helices 1 and 2, includes a single asparagine residue which, like GLUT 1, provides a potential site for N-linked glycosylation (Thorens *et al.*, 1988).

The glucose transport kinetics and cytochalasin B binding characteristics of GLUT 2 (Axelrod and Pilch, 1983) have indicated a distinct metabolic role for this protein in the tissues in which it is expressed. The erythrocyte/brain glucose transporter GLUT 1 has been shown to exhibit a high affinity for glucose having a K_m of 1-2mM (Wheeler and Hinkle,1985) whereas, in contrast, GLUT 2 exhibits a much lower affinity for its substrate having a K_m of 15 to 20mM (Craik and Elliot, 1979). Thus the K_m of GLUT 2 exceeds the physiological blood glucose concentration (5-10mM). These properties however are well suited to the physiological role of the liver with respect to whole body glucose homeostasis. The liver differs from most other organs of the body in that it can both take up and release glucose. Following a carbohydrate meal glucose levels in the blood rise and as a result the liver takes up glucose and converts it into glycogen. When blood glucose levels fall, glycogen is broken down and the resulting glucose is transported out of the liver, via GLUT 2, into the vascular system. Thus the high capacity of GLUT 2 ensures that the transport rate is never rate-limiting for glucose metabolism. Fluctuations in blood glucose concentrations are matched with rapid changes in the

intracellular glucose concentration thus facilitating the maintenance of glucose homeostasis.

Glucose absorption by epithelial cells of the intestine is a two step process. The Na^+/glucose co-transporter, localised in the brush border of the apical membrane, transports glucose from the lumen into the cell against its concentration gradient. Release of glucose across the basolateral membrane into the bloodstream is then mediated by GLUT 2.

Glucose uptake into the β–cells of the islets of Langerhans, in response to increases in the concentration of blood glucose, is the main physiological signal that induces insulin release. Glucokinase, a unique hexokinase isoenzyme with a high K_m for glucose (approximately 6mM) (Vischer *et al.*, 1987), is also expressed at high levels in β-cells. GLUT 2, by virtue of its high K_m for glucose, ensures that β-cell intracellular glucose concentrations rapidly change in response to changes in extracellular glucose concentration. However, it is has been suggested that glucokinase is the rate limiting enzyme in glucose metabolism and hence the "glucose sensing" mechanism which controls the conversion of glucose to the appropriate signal which induces insulin secretion (Meglasson and Matschinsky, 1984).

1.2.4 GLUT 3 - the brain-type glucose transporter

GLUT 3 was first cloned by Kayano *et al.* (1988). Using low-stringency hybridisation conditions to screen a human foetal skeletal muscle library with HepG2 glucose transporter cDNA, a transporter-like protein of 496 amino acids in size was identified. The protein was found to have 64.4% and 51.6% identity with the previously described human erythrocyte/HepG2 and liver-type glucose transporter sequences respectively (Kayano *et al.*, 1988). GLUT 3 mRNA, like that of GLUT 1, has been detected in a wide variety of tissues. However levels of GLUT 3 mRNA expression are highest in the brain, placenta and kidney (Kayano *et al.*, 1988).

Since the cloning of human GLUT 3, homologues have also been identified and cloned from mouse (Nagamatsu *et al.*, 1992), and chicken (White *et al.*, 1991). The human and mouse proteins are highly homologous, having 83% amino acid identity although substantial differences occur between species in the extracellular loops connecting transmembrane regions 1 and 2 and at the COOH -terminal domain regions (Nagamatsu *et al.*, 1992).

Western blotting studies, using antibodies raised to the COOH -terminal sequence of human GLUT 3, have shown that GLUT 3 protein is detectable in brain but not in human placenta or fat (Maher *et al.*, 1992). Shepherd *et al.* (1992) also reported the detection of low levels of GLUT 3 protein in human heart, placenta and liver and barely detectable levels in kidney. Analogous studies concerned with the distribution of GLUT 3 protein in rodent tissues have reported that GLUT 3 expression is more limited, with protein only being detectable in the brain (Maher *et al.*, 1992).

1.2.5 GLUT 4 - the insulin responsive glucose transporter

Insulin promotes a 20 to 30 -fold increase in the rate of glucose transport across the membranes of adipocytes. This insulin-stimulated increase results, at least in part, from the translocation of a latent pool of transporters from an intracellular location to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). In a series of studies in 3T3-L1 adipocytes, utilising anti-GLUT 1 antibodies, it was demonstrated that, upon insulin stimulation, GLUT 1 transporter protein was increased approximately 3 -fold at the plasma membrane whereas the rate of 2-deoxyglucose uptake increased 12 to 15 -fold. Thus the increased level of GLUT 1 protein at the plasma membrane could not fully account for the observed increase in 2-deoxyglucose uptake (Simpson and Cushman, 1986). Through the generation of a specific monoclonal antibody, IF8, which recognised a glucose transporter unique to fat and muscle (James *et al.* 1988), it was demonstrated that insulin stimulation of

adipocytes resulted in the translocation of the IF8-reactive transporter from an intracellular pool to the plasma membrane (Zorzano *et al.*, 1989). Moreover it was shown that this transporter species comprised the majority of the total transporter pool which was responsible for the increase in transporter number at the plasma membrane following insulin stimulation. Not long after this discovery, a number of research groups using low-stringency cross-hybridisation strategies and GLUT 1 cDNA probes, identified cDNAs encoding the "insulin-regulatable" glucose transporter isoform in human adipose and muscle tissue (Fukomoto *et al.*, 1989), rat heart (James *et al.*, 1989; Birnbaum, 1989), rat muscle (Charron *et al.*, 1989) and mouse 3T3-L1 adipocytes (Kaestner *at al.*, 1989). The newly identified glucose transporter which cross-reacted with IF8 was subsequently designated GLUT 4.

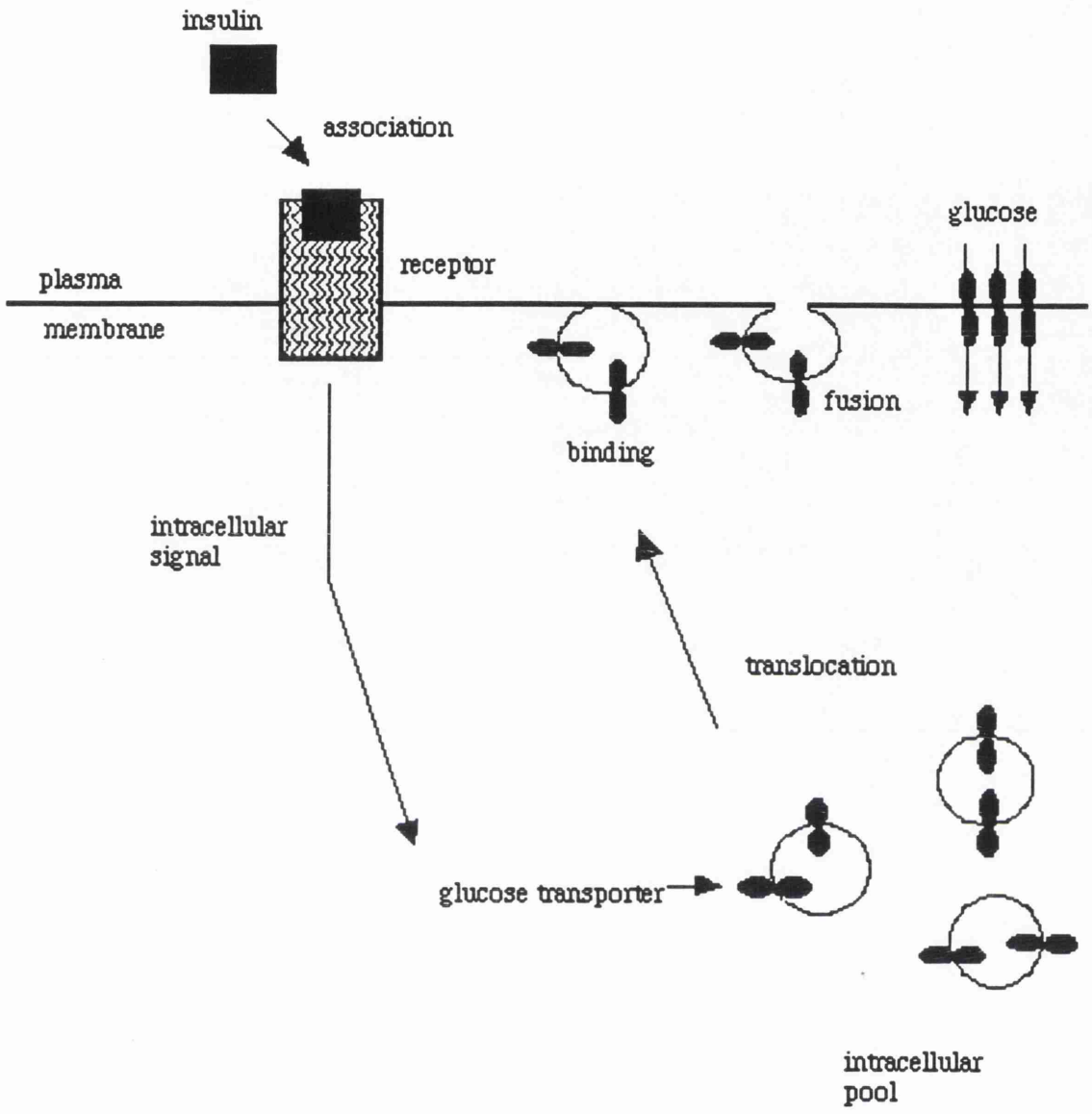
The sequence of GLUT 4 is highly conserved between species with 95% and 96% identity between sequences of human, rat, and mouse respectively. Human GLUT 4 is a 509 amino acid protein and has 65% identity with human GLUT 1, 54% identity with human GLUT 2 and 58% identity with human GLUT 3.

The three dimensional structure of GLUT 4 is predicted to be similar to that of GLUTs 1-3 although the protein has an extended NH_2 terminus relative to the other isoforms. In addition, the extracellular loop connecting transmembrane regions 1 and 2 is longer in GLUT 4 than in GLUTs 1 and 3. It could therefore be postulated that these structural differences in GLUT 4 relate to its unique insulin-responsiveness and targeting properties (see 1.5).

1.2.6 GLUT 5 -the intestinal glucose transporter

One of the more recent members of the facilitative glucose transporter family to have been identified and cloned is GLUT 5 (Kayano *et al.*, 1990). Using a low-stringency cross-hybridisation approach to screen a human jejunal cDNA library, a cDNA clone encoding a 501 amino acid glucose transporter-like protein was isolated (Kayano *et al.*, 1990). This protein is the most divergent member of the facilitative

FIGURE 1.2 Diagrammatic representation of insulin-stimulated translocation



Insulin, on binding to its receptor, generates an intracellular signal which promotes the translocation of GLUT 4 containing vesicles from an intracellular location to the plasma membrane. These vesicles fuse with the plasma membrane resulting in an increase in the number of functional GLUT 4 transporters at the cell surface. Glucose uptake into the cell is subsequently increased 15 to 20 -fold.

transporter family exhibiting 42, 40, 39, and 42% identity with human GLUT 1, GLUT 2, GLUT 3 and GLUT 4 respectively. Northern blot analysis has shown that GLUT 5 mRNA is detectable at high levels in the human small intestine, kidney, human testis and spermatozoa, and at lower levels in human muscle and adipose tissue (Kayano *et al.*, 1990). Western blotting and immunohistochemical studies have indicated that the protein is expressed in human muscle, human testis and spermatozoa, human adipose cell plasma membranes, small intestine, kidney and in the heart (Shepherd *et al.*, 1992; Burant *et al.*, 1992). In addition, more recently it has been demonstrated that GLUT 5 is also present within the endothelial cells lining brain microvasculature, although levels are approximately 4 -fold lower in whole brain relative to the kidney (Mantych *et al.*, 1993).

The precise role of GLUT 5 is not yet fully understood. However it has been postulated that, rather than mediating the uptake of glucose into cells, GLUT 5 may function primarily as a transporter of D-fructose. Studies carried out by Burant *et al.* (1992) demonstrated that when mRNA encoding GLUT 5 was micro-injected into *Xenopus* oocytes, an increase in D-fructose uptake was observed. However GLUT 5-expressing oocytes showed little ability to transport 2-deoxyglucose.

1.2.7 GLUT 6 -a pseudogene

A cDNA clone encoding glucose transporter-like sequence was recently identified in a human small intestine cDNA library and designated GLUT 6 (Kayano *et al.*, 1990). The gene however, although exhibiting a high percentage identity to GLUT 3 (76.9%), was found to be a pseudogene-like sequence, since its cDNA was found to contain multiple translation termination codons (Bell *et al.*, 1990).

1.2.8 GLUT 7 -the hepatic microsomal glucose transporter

When blood glucose levels are low, the liver releases glucose from glycogen, together with glucose formed from *de novo* synthesis, into the vascular system thus maintaining glucose homeostasis. The terminal step of both gluconeogenesis and glycogenolysis is catalysed by the glucose-6-phosphatase enzyme, the catalytic unit of which is located in the lumen of the endoplasmic reticulum. The route by which glucose, released by the glucose-6-phosphatase system, crossed the endoplasmic reticulum membrane remained unresolved until the identification of a specific endoplasmic reticulum glucose transporter, GLUT 7 (Waddell *et al.*, 1992). This transporter, which to date has only been cloned from rat, is a 528 amino acid residue protein with 68% identity to GLUT 2. Using specific antibodies to this isoform Waddell *et al.* (1991) demonstrated that the protein was expressed as a 52kDa protein in both rat and human liver microsomes. In addition it was demonstrated that the protein contained a COOH -terminal endoplasmic reticulum retention signal. Thus GLUT 7 functions in tandem with GLUT 2, with the former protein transporting glucose from the lumen of the endoplasmic reticulum into the cytosol and the latter transporting glucose across the plasma membrane into the circulation.

1.3 Insulin regulation of GLUT 4 translocation

Insulin stimulation of rat adipose cells, 3T3-L1 adipocytes and muscle cells results in a large stimulation of glucose uptake. In rat adipose cells stimulation with insulin produces an approximate 20 to 30 -fold increase in glucose transport (Ploug *et al.*, 1987) whereas in rat muscle cells uptake is increased 7 -fold (Whitesell and Abumrad, 1986). Kinetic evidence has shown that the major effect of insulin is to increase the V_{max} of glucose uptake (Ploug *et al.*, 1987; Dohm *et al.*, 1988; Whitesell and Abumrad, 1986; Palfreyman *et al.*, 1992).

In 1980 two groups showed, independently, that insulin stimulation of rat adipocytes resulted in an increase in glucose transporter number at the cell surface and moreover that this insulin-stimulated increase resulted from the movement of glucose transporters from an intracellular location to the plasma membrane - a process termed translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Zorzano *et al.* (1989) subsequently showed that insulin stimulation promoted the translocation of two transporter species in rat adipocytes, GLUT 1 and GLUT 4. Furthermore they demonstrated, by using antibodies specific for each transporter isoform, that GLUT 4 comprised the majority of the total intracellular pool, and that it was largely this isoform which accounted for the increase in transporter number at the cell surface following insulin stimulation.

Immunofluorescence and subcellular fractionation studies in murine 3T3-L1 adipocytes, carried out by Piper *et al.* (1991), showed that under basal conditions GLUT 1 was located both within intracellular vesicles and at the plasma membrane, but that GLUT 4 protein was predominantly located within intracellular vesicles. Insulin stimulation resulted in a 40% decrease in GLUT 4 protein and a 50% decrease in GLUT 1 protein intracellularly which was concomitant with a 10 fold and 3.5 -fold increase in the level of GLUT 4 and GLUT 1 respectively at the plasma membrane.

This intracellular sequestration and insulin induced redistribution of GLUT 4 was subsequently found to occur in other insulin-responsive tissues such as brown adipose tissue (Slot *et al.*, 1991a), skeletal muscle (Klip *et al.*, 1987; Hirshman *et al.*, 1990; Klip *et al.*, 1992; Lund *et al.*, 1993) and heart muscle (Watanabe *et al.*, 1984; Slot *et al.*, 1991b).

Since the pioneering studies of Suzuki and Kono (1980) and Cushman and Wardzala (1980), various strategies have been employed to determine the absolute levels of each transporter within intracellular and plasma membrane regions in both basal and insulin stimulated cells. As mentioned previously, insulin stimulation of adipocytes results in a substantial increase in glucose uptake concomitant with an increase in glucose transporter number at the cell surface. However results from

many studies have shown that there is a discrepancy between the number of glucose transporters recruited to the cell surface and the extent of glucose uptake observed in response to insulin. The glucose uptake observed in many studies has been shown to be greater than that which can be accounted for simply by the increase in glucose transporter number at the cell surface. This discrepancy however may be the result of the particular method employed to assess the number of transporters at the cell surface following insulin stimulation (see 1.4)

1.4 Methods to assess GLUT 4 translocation

1.4.1 Cytochalasin B

Cytochalasin B, a fungal metabolite, is able to bind reversibly to facilitative D-glucose transporters and in so doing is able to inhibit glucose transport. The binding of cytochalasin B to glucose transporters can be inhibited by the D- but not L-enantiomer of glucose and complete inhibition of binding can be achieved in the presence of D-glucose concentrations in the order of 500mM (Cushman and Wardzala, 1980). However the binding of cytochalasin B to cell membranes is complex since the substance is able to interact with additional non-facilitative glucose transporter binding sites such as cytoskeletal proteins (Shanahan, 1983). However in the presence of cytochalasin E, a related fungal metabolite, the binding of cytochalasin B to non-D-glucose facilitative glucose transporter protein binding sites can be reduced, since cytochalasin E interacts with cytoskeletal proteins but not glucose transporters (Shanahan, 1983).

Tritiated cytochalasin B has been widely used as a reagent to determine the number of facilitative glucose transporters in cell membranes. Baldwin *et al.* (1982) postulated that the fungal metabolite binds to transporter proteins with a stoichiometry of 1:1. This tritiated compound can be used to irreversibly photolabel glucose transporters since it can be cross-linked to the transporters by irradiation under U.V.

light (Shanahan, 1982; Carter-Su *et al.* 1982; Shanahan, 1983). Photolabelling is thought to occur through the photoactivation of an aromatic amino acid residue in the glucose transporter (Deziel *et al.*, 1984). A number of mutational studies have been carried out in an attempt to determine the cytochalasin B photolabelling site in glucose transporters (Garcia *et al.*, 1992; Katagiri *et al.*, 1991). However the precise site of interaction has not yet been defined although it has been postulated that the site lies within membrane spanning helices 10 and 11 (Cairns *et al.*, 1987). It was, by utilising tritiated cytochalasin B for binding studies in microsomal and plasma membrane fractions isolated from rat adipocytes, that Cushman and Wardzala (1980) first demonstrated the increase in glucose transporter number at the cell surface in rat adipocytes following insulin stimulation and hence translocation.

However it is important to note that this method also is inaccurate in determining the true extent of translocation since the measurement of cytochalasin B binding sites in plasma membrane or intracellular membranes relies on the membrane preparations being free from cross-contamination from other membrane fractions (see 1.4.3).

1.4.2 [^{3}H] ATB-BMPA

Holman and associates have recently developed ATB-BMPA (2-N-4 (1-azi-2,2,2-trifluoroethyl)benzoyl-1,3,-bis(D-mannos-4-yloxy)-2-propylamine), an affinity labelling reagent specific for glucose transporters. This compound is membrane impermeant and hence only labels glucose transporters which are located at the cell surface. The compound is a bis-mannose derivative in which the two mannose units are linked by a propyl bridge. Photolysis of this compound generates a highly reactive carbene through the loss of an N_2 from the diazirine, 4-(1-azi-2,2,2-trifluoroethyl)benzamido, functional group which is linked to the middle carbon of the mannose-linking propyl bridge.

This label has been used in a number of studies to measure the extent and rate of glucose transporter appearance at the plasma membrane following insulin stimulation (Holman *et al.*, 1990; Yang *et al.*, 1992; Calderhead *et al.*, 1990; Yang and Holman, 1993). Using ATB-BMPA to label cell surface glucose transporters in rat adipocytes it was demonstrated that, following insulin stimulation, GLUT 4 levels increased 15 to 20 -fold at the plasma membrane while GLUT 1 levels increased approximately 5 -fold (Holman *et al.*, 1990).

1.4.3 Subcellular fractionation of membranes and immunoblotting with glucose transporter-specific antibodies

Glucose transporter translocation is often assessed by preparing membrane fractions from basal and insulin-stimulated cells and immunoblotting these fractions with anti-GLUT 1 and anti-GLUT 4 antibodies. However this method frequently under estimates the extent of glucose transporter translocation due to difficulties in obtaining pure membrane fractions. For an accurate quantitation of the glucose transporter levels within these individual membrane fractions it is necessary for each fraction to be free from other contaminating membranes. It is often the case that during the preparation of plasma membranes by centrifugation these membranes become cross-contaminated with low density microsomal fractions. Hence, in the case of basal adipocytes, cross-contamination of plasma membranes with low density microsomal membranes, which contain high levels of GLUT 4, could lead to a significant under estimation of insulin-stimulated glucose transporter redistribution.

In one of the earliest studies carried out to assess the extent of GLUT 1 and GLUT 4 translocation in rat adipocytes, Zorzano *et al.*, (1980), prepared subcellular membrane fractions from basal and insulin-stimulated cells and demonstrated that insulin stimulation resulted in an 8 -fold increase in GLUT 4 in the plasma membrane and a 1.6 -fold increase in GLUT 1 protein. These results therefore could not fully

account for the 20 to 30 -fold increase in glucose uptake observed in response to insulin.

In contrast to these results Holman *et al.* (1990) demonstrated, using the ATB-BMPA glucose transporter specific label, that insulin stimulation of rat adipocytes resulted in a 15 to 20 -fold increase in GLUT 4 and a 5 -fold increase in GLUT 1 at the plasma membrane.

Thus immunoblotting of subcellular membrane fractions to assess transporter translocation sometimes grossly under estimates the true extent of translocation. A similar underestimation of translocation occurs using cytochalasin B binding and subcellular fractionation (see 1.4.1)

Immunoblotting specific membrane fractions with antibodies out-with their linear response range with respect to sample concentration can also lead to the inaccurate assessment of transporter translocation. This potential inaccuracy however can be dissipated by first subjecting samples of varying protein concentration to quantitative analysis. In addition, immunoblotting of membrane fractions should be carried out using saturating concentrations of each isoform-specific antibody to avoid possible affinity differences between antibodies leading to inaccurate quantitation of transporters.

1.4.4 Immunochemical techniques

Immunochemical techniques circumvent the need to prepare subcellular fractions from cells to assess glucose transporter translocation since they can be used in fixed whole cell preparations. These techniques therefore are more accurate in determining glucose transporter localisation within basal and insulin stimulated cells.

Piper *et al.* (1991), using confocal laser immunofluorescence microscopy demonstrated that in basal 3T3-L1 adipocytes GLUT 4 staining was predominantly perinuclear but that GLUT 1 staining occurred both within intracellular regions and at the plasma membrane. Insulin-dependent translocation of both glucose transporters

was indicated by a decrease in intracellular GLUT 1 staining and an increase in GLUT 4 staining at the plasma membrane. In addition, distinct intracellular compartments for GLUT 4 and GLUT 1 localisation were demonstrated by double immunofluorescent techniques using both fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated goat anti-rabbit antisera.

Slot *et al.* (1991a) used immunoelectron microscopy with protein A-gold antibodies to determine the localisation of GLUT 4 in basal and insulin stimulated rat brown adipose cells. These investigators demonstrated that 99% of GLUT 4 labelling occurred intracellularly in basal conditions but that following insulin stimulation 40% of the labelling occurred at the cell surface. In addition they observed GLUT 4 labelling in coated pits and in early endosomes after insulin treatment. These observations provided further support to previous reports suggesting that the major mechanism by which insulin increases glucose uptake is by the translocation of GLUT 4 protein. Furthermore, the staining reported in early endosomes and coated pits following insulin stimulation suggested that GLUT 4 protein undergoes recycling from the cell surface (see 1.6).

Therefore, in summary, the most accurate techniques available at present to determine the extent of translocation are approaches employing the glucose transporter specific label ATB-BMPA and immunocytochemical techniques. Both of these approaches are advantageous in that they circumvent the need to prepare subcellular membrane fractions by differential centrifugation. As mentioned previously membrane fractionation can lead to an under estimation of translocation if membrane cross-contamination occurs.

Immunocytochemical techniques are very sensitive in that they facilitate the detection of very low levels of transporter protein. An added advantage is that this technique can be applied to intact cells and consequently the precise location of the transporter proteins within the cell can be determined at any stage before or after insulin stimulation.

ATB-BMPA, the glucose transporter-specific membrane impermeant label can be used to assess the number of transporters localised intracellularly if the cells are subjected first to permeabilisation. Hence the both the total number of transporters and the number of transporters at the plasma membrane before and after insulin stimulation can be assessed.

These techniques are currently being employed in a wide range of studies and hence better experimental conditions are now emerging which allow more accurate translocation analyses to be made.

1.5 Glucose transporter targeting

GLUT 4 is unique compared to GLUTs 1-5 because of its efficient exclusion from the cell surface and sequestration in intracellular structures (Blok *et al.*, 1988; Orci *et al.*, 1989).

All facilitative glucose transporters which have been cloned to date are proposed to be structurally similar, comprising 12 α–helical membrane spanning regions with the NH_2 and COOH -termini both intracellularly localised. Amino acid diversity between isoforms does however occur in the extracellular loop, between transmembrane regions 1 and 2, and in the intracellular loop between transmembrane regions 6 and 7. In addition diversity occurs at the COOH terminus and the NH_2 terminus, where GLUT 4 is extended by several residues relative to other glucose transporter isoforms. Hence it is likely that these regions of diversity between isoforms contain information that is recognised by cytosolic machinery to determine the eventual destination of the protein in the cell.

Many investigations have been carried out in an effort to determine if a targeting motif exists in the GLUT 4 sequence which accounts for its intracellular sequestration. To elucidate the targeting mechanisms of GLUT 4, two main approaches have been taken. The first approach has involved expressing GLUT 1-GLUT 4 chimeric molecules in various cell types to assess whether the molecules are

targeted to intracellular regions, like GLUT 4, or whether they are targeted to the plasma membrane like GLUT 1 (Asano *et al.*, 1992; Piper *et al.*, 1992). Piper *et al.* (1992), by transiently expressing GLUT 1-GLUT 4 chimeric transporters in chinese hamster ovary (CHO) cells, showed that substitution of the NH_2 terminus of GLUT 1 with that of GLUT 4 resulted in a marked intracellular sequestration of GLUT 1 protein, and conversely that substitution of the GLUT 4 amino-terminal region with that of GLUT 1 abolished the efficient intracellular sequestration of GLUT 4. Hence from these results these investigators proposed that the cytoplasmic portion of the NH_2 terminus of GLUT 4 is both necessary and sufficient for intracellular sequestration. In contrast to these results, Asano *et al.* (1992), by stably expressing GLUT 1-GLUT 4 chimeric glucose transporters in CHO cells, reported that two domains of GLUT 4, that were neither the NH_2 or COOH -terminal domain, dictated intracellular sequestration. The first domain included transmembrane spanning helices 2 and 3 and contained a leucine zipper motif, a specific sequence which has been postulated to contribute to the formation of a glucose transporter dimeric structure (White and Weber, 1989; Pessino *et al.*, 1991). The second domain was localised to transmembrane regions 7 and 8 of GLUT 4 (Asano *et al.*, 1992).

As a second approach to study GLUT 4 targeting Piper *et al.* (1993) constructed GLUT 4 proteins in which the NH_2 terminal portion of the protein contained amino acid deletions or substitutions. Transfection of CHO cells with the mutant proteins revealed that deletion of the first 8 amino acids from the NH_2 terminus or substitution of alanine for phenylalanine at position 5 in GLUT 4 resulted in a marked accumulation of the transporter at the cell surface. In addition it was reported that mutations at other amino acids surrounding phenylalanine 5 resulted in an increase in GLUT 4 cell surface expression but not to the same extent as the position 5 phenylalanine mutation. Thus the investigators concluded that the NH_2 terminus of GLUT 4 was necessary for intracellular sequestration (Piper *et al.*, 1992). Further to these findings James and Piper (1993) reported that the NH_2 terminal of GLUT 4 shares homology with endocytic signal sequences in cell surface

receptors. However they were of the opinion that the exclusion of GLUT 4 from the cell surface was not entirely mediated by the rapid endocytosis of GLUT 4 from the cell surface, since other investigators have reported that in fibroblasts 40% of the transferrin receptor, a protein with a similar internalisation motif, is present at the cell surface (Jing *et al.*, 1990). Therefore they proposed that the effective exclusion of GLUT 4 from the cell surface was due both to its retention in intracellular vesicles and its efficient internalisation through endocytosis pathways.

Other groups have previously reported that glucose transporter targeting in transfected cells is similar to targeting in native cells. Results from studies carried out in CHO, HepG2 or 3T3-L1 fibroblasts with GLUT 1 and GLUT 4 cDNA have shown that protein targeting was to the plasma membrane and intracellular regions respectively (Shibasaki *et al.*, 1992; Hudson *et al.*, 1992; Robinson *et al.*, 1992). Hence these studies alone have suggested that GLUT 4 targeting is dictated by the transporter protein and thus is independent of cell type. However it is important to note that due to the conflicting results of Asano *et al.* (1992) and Piper *et al.* (1992) it seems likely that it is not a single domain that dictates GLUT 4 protein targeting in cells but that perhaps domain-domain interactions are intimately involved.

The major difference between the chimeric studies carried out by Asano *et al.* (1992) and Piper *et al.* (1992) was that the former group used a stable expression system whereas the latter group used a transient transfection system. Therefore it could be postulated that the conflicting results obtained were a direct result of the types of expression systems used. It may have been the case that the transient system favoured the use of the NH_2 terminal internalisation motif whereas the stably produced transporter relied upon internal transporter sequences for targeting. Hence characterisation of the GLUT 4 intracellular targeting motif still remains to be defined.

In addition to determining the intracellular targeting domain of GLUT 4 it is also of considerable importance to determine if a specific region of GLUT 4 exists which dictates GLUT 4 translocation. It could be postulated that the domain which

dictates translocation is a distinct domain to that which dictates targeting. It is possible that a specific domain is present in GLUT 4 which facilitates the interaction of the transporter with specific intracellular factors, present in the cell cytosol or within the distinct GLUT 4 vesicle compartment, which are integral to the translocation process. Previous studies have shown that when GLUT 4 is expressed in various cell types it is targeted to an intracellular region as in its native adipocyte cell (Shibasaki *et al.*,1992; Haney *et al.*,1991; Robinson *et al.*, 1992). However when these cells are stimulated with insulin, transfected GLUT 4 translocation to the plasma membrane could not be demonstrated. Thus from these results it could be postulated that GLUT 4 translocation requires specific factors which are present only in adipocytes and muscle cells, cells in which GLUT 4 is a native transporter.

The specific intracellular vesicle population to which GLUT 4 protein is localised in adipocytes remains to be defined. Upon insulin stimulation only a small percentage of intracellular GLUT 4 undergoes translocation to the plasma membrane. Therefore it may be the case that only a small subset of GLUT 4 vesicles are competent for translocation. It could be suggested that in non-insulin sensitive GLUT 4-transfected cells the vesicle population to which the GLUT 4 is directed differs from the population of GLUT 4-containing vesicles in adipocytes. Hence it may be that GLUT 4-transfected cells do not exhibit translocation upon insulin stimulation because the vesicles in which GLUT 4 is located lack those specific factors necessary for translocation to occur.

GLUT 4 appears to be targeted to specialised intracellular vesicles in fat and muscle cells, a vesicular population distinct from that of GLUT 1 (Piper *et al.*, 1991). In adipocytes GLUT 4 has been localised to a vesicular population in which a specific synaptic vesicle protein, synaptobrevin is also expressed (Cain *et al.*, 1992). Several vesicle proteins have been implicated in the recycling and translocation of GLUT 4 (see 1.6).

1.6 Glucose transporter translocation and recycling

As mentioned in previous sections, the acute regulation of glucose transport by insulin in its target tissues, fat, muscle and heart, is now known to involve the specific insulin-regulatable glucose transporter isoform GLUT 4 (see 1.3). It is the ability of the GLUT 4 isoform to remain localised in the cytoplasmic tubulo-vesicular system in the basal state which allows GLUT 4 containing cells to increase glucose transport activity following insulin stimulation. It has been hypothesised that the intracellular sequestration of GLUT 4 in basal cells may be possibly due to either its efficient rapid removal from the cell surface or due to its slow rate of exocytosis (Piper *et al.*, 1992).

Slot *et al.* (1991a), using an immunocytochemical approach to study the effects on GLUT 4 trafficking in brown adipose tissue, reported that upon insulin stimulation 40% of the GLUT 4 labelling was at the cell surface. In addition, coated pits and early endosomes were enriched with GLUT 4. Together these observations suggested that GLUT 4 recycled via the coated-pit endosome pathway and that the main effect of insulin was to increase GLUT 4 exocytosis from tubulo-vesicular structures to the plasma membrane.

Since this investigation various studies have been carried out in an attempt to determine the kinetics of GLUT 4 exocytosis and to compare its trafficking properties with those of GLUT 1 (Jhun *et al.*, 1992; Palfreyman *et al.*, 1992; Yang *et al.*, 1992; Yang and Holman, 1993). As previously mentioned 3T3-L1 adipocytes express the GLUT 1 transporter isoform, in addition to GLUT 4, and both transporters undergo translocation in response to insulin (Zorzano *et al.*, 1989), however the extent of translocation observed for GLUT 1 is considerably less than that observed for GLUT 4 (Calderhead *et al.*, 1990).

Yang and Holman (1993) showed, using ATB-BMPA to label cell surface GLUT 1 and GLUT 4 in 3T3-L1 adipocytes, that both glucose transporters were constantly recycled between the plasma membrane and low density microsomes in the

presence and absence of insulin. The rate constants for the endocytosis and exocytosis of GLUT 1 and GLUT 4 in 3T3-L1 cells were determined when the cells were maintained in the presence of insulin or in the basal state. It was calculated that the endocytosis rates in basal and insulin-stimulated cells were similar for both GLUT 1 and GLUT 4 proteins. The major difference between the trafficking of GLUT 4 and GLUT 1 however was found to be the much slower exocytosis rate of GLUT 4 in the basal state. Insulin stimulation resulted in an increase in the rate of glucose transporter exocytosis with the exocytosis rate of GLUT 4 being 8.6 -fold faster in insulin-stimulated cells compared with basal cells (Yang and Holman, 1993).

These results were in contrast to those obtained by Jhun *et al.* (1992) who demonstrated that insulin increased cell surface GLUT 4 in rat adipocytes by modulating both the internalisation and externalisation steps of constituitively recycling GLUT 4. These investigators showed, by labelling rat adipocyte cell surface glucose transporters with an impermeable photoreactive glucose analogue B3GL (1,3-bis-(3-deoxy-D-glucopyranose-3-yloxy)-2-propyl 4 benzoyl-benzoate), that insulin reduced GLUT 4 endocytosis by 2.8 -fold and increased exocytosis by 3.3 -fold.

It is not yet apparent why Yang and Holman (1993) and Jhun *et al.* (1992) obtained differing results from these studies on the effect of insulin on GLUT 4 endo/exocytosis. It could be suggested that the conflicting results arose due to the different transporter-specific labels which were employed in each study, however further work will be required to resolve this current problem.

In other studies using the specific glucose transporter label ATB-BMPA, it was demonstrated that in 3T3-L1 cells GLUT 1 and GLUT 4 appear rapidly at the cell surface following insulin treatment with half times of approximately 2 min. However a lag of approximately 1 min was shown to occur between the appearance of transporters at the cell surface and the stimulation of glucose transport. This lag was thought to reflect the time required for transporters to dissociate from putative

trafficking proteins (Yang *et al.*, 1992). In addition it was demonstrated that the losses of cell surface GLUT 1 and GLUT 4 occurred with half times of approximately 12 min for each, which correlated well with the reduction observed in glucose transport. These results suggest that the larger insulin-stimulation of cell-surface availability of GLUT 4 (15-20 fold) compared with GLUT 1 (3 to 5 fold) was indeed due to the lower rate of GLUT 4 exocytosis in the basal state. These results therefore are in agreement with those of Yang and Holman (1993).

From immunofluorescence and immunolocalisation studies it has become apparent that in both rat and 3T3-L1 adipocytes GLUT 1 and GLUT 4 are differentially sorted within the cell (Piper *et al.*, 1991; Zorzano *et al.*, 1989). The transporters occupy distinct intracellular compartments although a degree of overlap between the two isoforms has been observed (Zorzano *et al.*, 1989). In a recent study the differential sorting of GLUT 1 and GLUT 4 in 3T3-L1 adipocytes was confirmed employing the vicinal-inactivating reagent phenylarsine oxide. This reagent was found to selectively block the exocytosis of GLUT 4 but not that of GLUT 1 (Yang *et al.*, 1992). Hence these results, taken together with the evidence that insulin increases the exocytosis rate both of GLUT 4 and GLUT 1, but that the rate for GLUT 4 is greater, suggests that the vesicle population enriched in GLUT 4 is distinct and may contain specific proteins which are involved in the specific transport of GLUT 4 to the plasma membrane.

Recent studies, examining the protein content of GLUT 4 vesicles, have identified various protein components which may have a role to play in the translocation and recycling of GLUT 4 protein. The cycle, involving GLUT 4 exocytosis followed by internalisation through the coated pit endocytic pathway, is very similar to the cycle proposed to occur for neurotransmitter-containing synaptic vesicles localised in nerve terminals (Trimble *et al.*, 1991; De Camilli and Jahn, 1990). The components of these synaptic vesicles are many, but a number of vesicle proteins including synaptophysin, Rab3A, p29 and synaptobrevin have been recently isolated. Several of these proteins have also been detected in the synaptic-like

microvesicles of endocrine cells, structures which resemble the GLUT 4-containing vesicles of adipocytes (Trimble *et al.*, 1991; Navone *et al.*, 1986). Using an immunoadsorption approach, it was recently demonstrated that one of these synaptic vesicle proteins, VAMP (synaptobrevin - vesicle associated membrane protein), is localised in the GLUT 4-containing vesicles of rat adipocytes (Cain *et al.*, 1992). In this study it was shown that VAMPs were components of the GLUT 4 vesicles and that, following insulin treatment, they too were translocated to the plasma membrane.

Secretory carrier membrane proteins (SCAMPs), proteins which constitute markers of general cell surface recycling systems have also been demonstrated to co-localise with GLUT 4 and VAMPs in low density microsomal vesicles in adipocytes (Laurie *et al.*, 1993). Using immunochemical and immunocytochemical approaches, it was shown that all three proteins co-localised in basal adipocytes and that SCAMPs constituted a major component of GLUT 4-containing vesicles. Membrane fractionation studies showed however that upon insulin stimulation, although a decrease in GLUT 4 and VAMPs was observed in the low density microsomal fraction, no change in the level of SCAMPs occurred. This partial segregation of VAMPs, SCAMPs and GLUT 4 was proposed to occur during sorting at the cell surface where SCAMPs were more efficiently sequestered and re-internalised.

It is clear that the recycling and translocation of GLUT 4-containing vesicles involves a number of proteins and more GLUT 4-vesicle membrane components probably remain to be identified. Hopefully identification of these proteins will lead to a better understanding of GLUT 4 trafficking and translocation.

1.7 The aims of this study

At the time when this study was undertaken, Northern blot analysis of GLUT 3 expression in human tissues had shown that GLUT 3 was expressed at highest levels in the brain. Its mRNA however was also detectable, albeit at lower levels, in adipose tissue, liver and muscle, tissues which are all insulin sensitive. These

findings suggested a possible role for GLUT 3 in the transport of glucose across the plasma membrane of neural and glial cells, and moreover that this isoform could be important in regulating glucose disposal in insulin-sensitive tissues in response to insulin. Similar Northern blot studies carried out in rat and mouse tissues in contrast showed that GLUT 3 mRNA had a more restricted tissue distribution compared with human tissues and that it was expressed primarily in brain.

The subsequent generation of an anti-peptide antibody to the human homologue of GLUT 3 has allowed investigators to determine the distribution of GLUT 3 protein in human tissues. Results of such studies have indicated that, although GLUT 3 levels are highest in brain, GLUT 3 protein is detectable in heart, placenta and liver, but not fat and muscle (Shepherd *et al.*, 1992).

Since studies of peripheral insulin resistance routinely utilise rodent models of diabetes and/or obesity, one of the aims of this study was to determine the distribution of GLUT 3 protein in mouse and rat tissues and to speculate on the potential role of this protein in whole body glucose homeostasis. As a first step in studying GLUT 3 protein distribution in mouse tissues, an anti-peptide antibody against the mouse GLUT 3 protein was generated. The successful generation of such an antibody thus allowed GLUT 3 protein distribution in mouse tissues to be determined and allowed expression patterns between mouse and human tissues to be compared.

In situ hybridisation analysis studies in mouse brain have also indicated that the expression of this isoform is not homogeneous throughout all regions of the brain (Nagamatsu *et al.*, 1992) which may be taken to imply that GLUT 3 protein levels between brain regions may also differ. Western blot studies carried out on selected regions of rat brain have indicated that, although the mRNA for GLUT 3 is detectable in most regions of the brain, the protein is absent from particular regions (Maher *et al.*, 1992).

Having shown, in the initial part of the study, that the anti-peptide antibody raised to mouse GLUT 3 recognised GLUT 3 protein in mouse tissues, the antibody

was then used to examine the levels of GLUT 3 protein in various regions within the rat brain. It was demonstrated that the antibody cross-reacted with rat GLUT 3 protein which was not surprising since the sequences of both mouse and rat GLUT 3 proteins are similar. Since most studies in brain have focused on GLUT 3 and GLUT 1 expression, the aim of this part of the study was to determine whether other glucose transporter protein species were expressed in brain regions. Previous studies have shown that the activity in areas of the brain, particularly those involved in the regulation of feeding, can be influenced by glucose, free fatty acids and insulin (Oomura, 1976). Hence, on the basis of these findings, it could be postulated that other glucose transporter isoforms, whose activity could be regulated by hormones such as insulin, and which could be involved in monitoring glucose levels, may also be expressed in the brain. Therefore to examine the distribution of different glucose transporters in rat brain, individually dissected regions of the brain were immunoblotted with a panel of anti-peptide antibodies specific for GLUTs 1-4.

The majority of this study however was concerned with elucidating those factors which regulate the insulin-responsiveness of glucose transporters and their ability to translocate following insulin stimulation. GLUT 2 and GLUT 4 are both facilitative glucose transporters expressed in insulin-responsive tissues, liver and fat respectively. Insulin stimulation of adipocytes results in a rapid and large 20 to 30 -fold increase in glucose uptake. Several groups have shown independently that it is the redistribution of GLUT 4 protein from an unidentified intracellular region to the plasma membrane which accounts for most, if not all, of this insulin-stimulated increase in glucose uptake (Suzuki and Kono, 1980; Cushman and Wardzala, 1980; Slot *et al.*, 1991a).

GLUT 2 is localised to the plasma membrane of hepatocyte cells and is absent from intracellular membranes (Thorens *et al.*, 1990). Insulin stimulation of resting hepatocytes, in contrast to adipocyte cells, does not stimulate glucose transport and by inference GLUT 2 does not undergo insulin-induced translocation (Thorens *et al.*, 1988; Fukomoto *et al.*, 1988).

The main part of the investigation described herein was concerned with determining whether GLUT 2 and GLUT 4, when expressed in "foreign" cells, maintained their native properties or whether their properties were influenced by their cellular environment. In other words the aim of this study was to determine whether GLUT 2, when expressed in a fat cell environment could undergo translocation in response to insulin and, complementary to this, whether GLUT 4 when expressed in a liver-type cellular environment still exhibited translocation following insulin stimulation. The results from such an investigation should help determine whether the specific properties of glucose transporter proteins are inherent in the protein sequence and/or whether cellular environment influences the properties of individual transporters. Previous studies, investigating the properties of GLUT 4 in GLUT 4-transfected cells, have demonstrated that, although the protein is targeted correctly to intracellular membranes, the protein does not exhibit translocation to the plasma membrane following insulin stimulation (Shibasaki *et al.*, 1992). Consequently it has been postulated that GLUT 4 protein itself probably contains the information necessary for its correct targeting in cells but that specific cellular factors, present in native GLUT 4-expressing cells, are both necessary and essential for the translocation process. Therefore it may be interesting to determine if GLUT 2 in a fat cell environment could be induced to undergo translocation in response to insulin. Should the results from this study indicate that GLUT 2 does not undergo translocation in response to insulin then it would seem probable that translocation is a unique property to GLUT 4 that maybe is dictated by both transporter sequence and cellular environment.

It is hoped that results from this study will contribute to an understanding of the process of translocation, however it is quite possible that the process is governed by many factors and is not merely dictated by glucose transporter protein sequence and/or cellular environment.

CHAPTER 2

MATERIALS AND GENERAL METHODS

Unless stated otherwise, all materials were obtained from Sigma Chemical Company (Poole, Dorset, U. K.).

2.1 ANTIBODY PREPARATIONS

The following procedure was carried out by Dr. Gwyn Gould and animal house technical staff from the Department of Biochemistry, University of Glasgow.

2.1.1 Antisera production and preparation of KLH-coupled peptide

Peptides corresponding to the C-terminal 13 amino acids of GLUTs 2-5 were individually coupled to keyhole limpet haemocyanin via Sulpho-m-Maleimidobenzoyl-N-hydroxysulfosuccinimide (Sulpho-MBS), utilising an N-terminal cysteine residue incorporated into the peptide (Lerner, 1981).

20mg of keyhole limpet haemocyanin was dissolved in 2ml of 50mM sodium phosphate buffer and dialysed overnight against the same buffer to remove any amines present. Following this, 17µl of 300mM N-ethylmaleimide was added (5µmoles) and the mixture was incubated at room temperature for 30 min. 6.2mg of Sulpho-MBS was then added (15 µmoles) and the mixture was again incubated at room temperature for 30 min. The pH was adjusted to pH 6.0 with addition of 1N HCl and the mixture was dialysed overnight at 4°C against 20mM sodium phosphate/135mM NaCl, pH 5.6, to remove unreacted or hydrolysed Sulpho-MBS.

The following morning an equal volume of 20mM sodium phosphate/150mM Na Cl, pH 8.0 was added to the diasylate (the pH was quickly adjusted with 1 N HCl or 1N NaOH to a pH of 6.7). Immediately thereafter, approximately 9mg of solid

peptide (6 μmoles) was added to the mixture and dissolved by vigorous vortexing.The reaction was then incubated at room temperature for 30 min.

Coupling was determined by dialysing a small sample in 4mm wide Spectra/Por 2, MW CO 13,000 tubing against 10mM Na phoshate/150mM NaCl, pH 7.4, in the cold for 72 h. The concentration of the sample was determined before and after this latter procedure.

The complete procedure for the coupling of thiol peptides to KLH typically yielded 2.5mg/ml peptide with approximately 50% of this representing coupled peptide.

Rabbits were immunised at multiple intradermal sites with 250μg of conjugated peptide in 1ml of a 40/60 mixture of phosphate buffered saline (PBS: 150mM NaCl, 10mM $NaH_2PO_4.2H_20$ (Na phosphate), pH 7.4) /Freunds complete adjuvant. One month later, animals were boosted with the same, but using incomplete Freunds adjuvant. Two weeks later, blood was collected and antibodies were affinity purified by antigen specificity using the Pierce Immunopure system (see 2.1.2). Booster injections were repeated and serum was harvested every second month for three further bleeds. Serum was then stored in aliquots at -80°C until required.

2.1.2 Purification of anti-peptide antibodies

0.5ml of 250mM Na phosphate, pH 6.5 was added to 5ml of anti-GLUT peptide rabbit serum and this was applied to a protein A sepharose column which had been pre-washed with 10ml of 25mM Na phosphate, pH 6.5. The serum was allowed to run through the column and was re-applied a further 4 times. The column was then washed with 25mM Na phosphate, pH 6.5 until the eluant gave an O.D. reading at 280nm of less than 0.1. The column was allowed to almost run dry before the slow addition of 10ml of 0.1M glycine, pH 2.4 to elute the bound IgG antibodies. The eluant was then collected in 1ml fractions and O.D. readings at

280nm were taken for every fraction. Those fractions with the highest O.D. readings were pooled and 50µl of 0.5M Tris pH 6.8 was used added to neutralise each sample. The samples were then dialysed against PBS, pH 7.4 overnight at 4°C. After dialysis, the sample was applied to an affinity column which consisted of Sepharose beads to which the antigenic peptide was covalently attatched (Pierce Immunopure antigen/antibody Immobilisation Kit 2). The column was then incubated at room temperature for 90 min to allow those antibodies which recognised the Sepharose-conjugated peptide to bind. The column was then washed with PBS, pH 7.4 until the eluant gave an O.D. reading at 280nm of zero. The specific antibody was eluted with Pierce Immunopure IgG elution buffer (pH 2.5), collected in 1ml fractions and the 0.D. reading at 280nm determined for each fraction. 50µl of 1M Tris, pH 6.8 was then added to those fractions with the highest O.D. readings. These were pooled and dialysed overnight against PBS, pH 7.4 at 4°C. Finally, dialysed samples were aliquoted into 1.5ml microfuge tubes, snap frozen in liquid nitrogen and stored at -80°C.

2.2 PROTEIN ASSAY

Protein concentrations were determined by the method of Peterson (1977) adapted from the method of Lowry *et al.* (1951).

2.2.1.Preparation of reagents for protein assay

The following 3 solutions were prepared and stored at room temperature until required:-

Solution 1: 2mM $CuSO_4.5H_2O$, 3.5mM potassium tartrate, and 476mM Na_2CO_3 in H_20.

Solution 2 : 10% (w/v) SDS in H_2O.

Solution 3 : 0.8N NaOH in H_2O.

"Reagent A" was then prepared by combining equal volumes of solutions 1, 2, 3 and distilled H_2O.

2.2.2 Protein determination

A sample containing between 1-20µg of protein was made up to 1ml with distilled H_2O in a glass test-tube and 0.1ml of 0.15% deoxycholate (15% (w/v) in H_2O) was added. After incubation at room temperature for 10 min, proteins were precipitated by the addition of 100µl of 72% (w/v) trichloroacetic acid. The samples were then centrifuged at 3000 x g for 15 min. The supernatant was discarded and the pellet allowed to dry at room temperature.

400µl of distilled H_20 and 400µl of Reagent A were added to solubilise the proteins and the samples incubated at room temperature for 10 min. 200µl of Reagent B (1:4 (v/v) Fisher Folin-Ciocalteu phenol reagent/H_2O) [Fisons Scientific equipment, England] was then added and the solution was vortexed immediately. After incubation at room temperature for 30 min, the absorbances of the reactions were read at 750nm, and the concentration of the unknown protein samples determined by comparison with the absorbances of the calibration graph prepared using 0, 1, 2, 5, 10, 12, 15, and 20µg of BSA.

2.3 SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS/polyacrylamide gel electrophoresis was carried out using 0.75, or 1.5mm thick vertical slab gels containing either 10% or 12.5% acrylamide, prepared by the method of Laemmli (1970). All reagents were of electrophoresis grade.

Slab gels were prepared using either Bio-Rad mini-PROTEAN II or Hoefer large gel units, with stacking gel of 2 and 5cm respectively. These consisted of 5% acrylamide/0.136% bisacrylamide in 125mM Tris-HCl, pH 6.8, 0.1% SDS,

polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) tetramethylethylenediamine (TEMED). The separating gels were 10 or 12.5% acrylamide/0.28 % bisacrylamide in 0.383mM Tris-HCl, pH 8.8, 0.1% SDS; polymerised with 0.1% (w/v) ammonium persulphate and 0.019% (v/v) TEMED.

Protein samples were solubilised in 1X sample buffer [93mM Tris-HCl pH 6.8, 10mM $Na_4P_2O_7$, 10mM EDTA, 2% (w/v) SDS, 20mM DTT, 10% (v/v) glycerol, 0.007% (w/v) bromophenol blue], vortexed to dissolve and stored at -80°C. Pre-stained molecular weight markers were used routinely [M_r 49,500-205,000 kilodaltons (Bio-Rad)].

Electrophoresis was carried out using 120mM Tris, 40mM glycine, 0.1% (w/v) SDS buffer (pH 8.3). Large gels were electrophoresed overnight using the Hoefer electrophoresis cell, and an LKB Bromma 2197 power supply, at 60-70 V per electrophoresis cell. Mini-gels were electrophoresed using the Bio-Rad mini PROTEAN II system at a constant voltage of 150V through the stacking gel and 180V through the separating gel. Gels were electrophoresed until the tracking dye had migrated to the bottom of the gel and good separaration of the molecular weight markers obtained.

2.4 WESTERN BLOTTING OF PROTEINS

Proteins were separated by SDS/polyacrylamide gel electrophoresis as previously described. The gels were removed from the plates and equilibrated in 25mM Na phosphate buffer, pH 6.5 (transfer buffer) at room temperature for 30 min. Each gel was then placed on top of an equal-sized sheet of nitrocellulose (0.45μm pore size, Schleicher and Schuell), pre-wet with transfer buffer, and then placed between 2 layers of 3mm filter paper also pre-wet with transfer buffer. The "sandwich" was then inserted between the plates of the gel holder cassette and transfer was performed using a Bio-Rad trans-blot or Bio-Rad mini trans-blot

electrophoretic transfer cell at a constant current of 275mA for 3 h at room temperature. The nitrocellulose membranes were then removed from the transfer cassette and the efficiency of transfer qualitatively determined by the presence and intensity of pre-stained molecular weight standards (Bio-Rad).

2.5 BLOCKING OF NITROCELLULOSE MEMBRANES AND PROBING WITH ANTI-GLUT ANTISERA

2.5.1 Blocking of nitrocellulose membranes and probing with anti-GLUT 2, 3, 4, or 5 antisera.

Non-specific binding sites on the nitrocellulose membranes were blocked by shaking for 30 min in 5% (w/v) non-fat milk [Marvel, Premier Brands U.K.] /1st wash buffer (150mM NaCl, 5mM Na phosphate, 0.98mM EDTA, 0.09% Triton X-100). The nitrocellulose was then transfered to 1% (w/v) non-fat milk/1st wash buffer containing antibody at 10μg/ml and incubated at room temperature overnight with shaking. After washing membranes four times, at 10 min intervals with 1st wash buffer, the membranes were incubated in 1% non-fat milk/1st wash buffer containing either ^{125}I-labelled goat anti-rabbit IgG (1μCi/20ml) [NEN Research Products, Du Pont) or HRP-linked goat anti-rabbit IgG [Amersham, PLC] at a dilution of 1:1000 for 3 h and 1 h respectively (see 2.6.1 and 2.6.2).

2.5.2 Blocking of nitrocellulose membranes and probing with anti-GLUT 1 antiserum

Non-specific binding sites on the nitrocellulose were blocked by shaking for 30 min in 3% BSA (w/v)/1st wash buffer. The nitrocellulose was then transfered to 3% BSA (w/v)/1st wash buffer containing GLUT 1 antibody at 10μg/ml and

incubated at room temperature overnight with shaking. After washing four times at 10 min intervals with 1st wash buffer, the membranes were incubated in 3% BSA (w/v)/1st wash buffer containing either ^{125}I-labelled goat anti-rabbit IgG (1μCi/20ml) or HRP-linked goat anti-rabbit IgG at a dilution of 1:1000 for 3 and 1 h respectively (see 2.6.1 and 2.6.2). Membranes were then washed four times at 10 min intervals with 2nd wash buffer (620mM NaCl/1st wash buffer), and, after drying between cellophane or incubating in ECL detection reagents, were exposed to Kodak film in an autoradiography cassette with a single high-speed intensifying screen. Films were then exposed at -80°C for 12-24 h or at room temperature for 10 seconds to 1hour for those membranes incubated with iodinated or HRP-linked secondary antibodies respectively, and developed using an X-OMAT processor.

2.6 IMMUNODETECTION OF PROTEINS

2.6.1 Immunodetection of proteins using Western blotting and autoradiography

Samples were electrophoresed on 10% or 12.5% polyacrylamide gels, proteins transferred to nitrocellulose membranes and membranes blocked as previously described. After incubating in primary antibody overnight, membranes were washed four times at 10 min intervals with 1st wash buffer. Following this, membranes probed with antisera to GLUTs 2-5 were incubated in 1% non-fat milk/1st wash buffer containing 1μCi/20ml ^{125}I-labelled goat anti-rabbit IgG, while membranes probed with anti-GLUT 1 antiserum were incubated in 3% BSA/1st was buffer containing ^{125}I-labelled goat anti-rabbit IgG. After incubation at room temperature for 3 hours the membranes were washed four times at 10 min intervals with 2nd wash buffer (620mM NaCl/1st wash buffer), draining off the final wash. The membranes were then dried overnight between 2 sheets of Bio-Rad cellophane,

mounted on cardboard and exposed to Kodak film for 12-24 h at -80°C. The film was then developed using the X-OMAT processor.

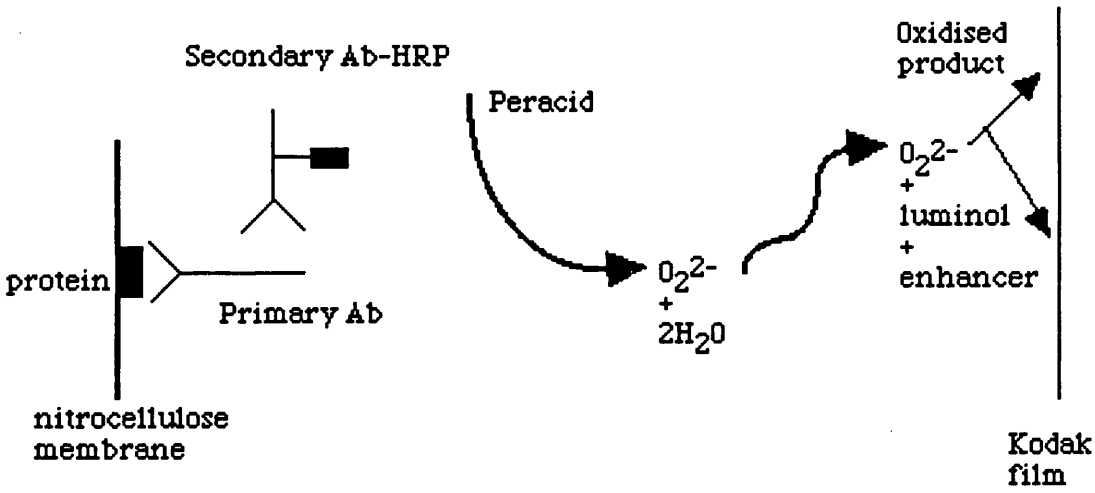
2.6.2 Immunodetection of proteins by Western blotting and the ECL detection system

Enhanced chemiluminescence (ECL) is a light emitting non-radioactive method for the detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. Horseradish peroxidase is used to catalyse the oxidation of luminol in the presence of hydrogen peroxide. Immediately after the oxidation, the luminol is in an excited state which decays to ground state via a light emitting pathway (see Fig. 2.1).

Samples were separated on 10% polyacrylamide gels and proteins transferred to nitrocellulose membranes as previously described (see 2.3 and 2.5). The membranes were then incubated with primary antibody overnight. After washing four times at 10 min intervals with 1st wash buffer, membranes probed with antisera to GLUTs 2-5 were incubated in 1% non-fat milk/1st wash buffer containing goat anti-rabbit HRP-linked secondary antibody, whilst membranes probed with anti-GLUT 1 antibodies were incubated in 3% BSA/1st wash buffer containing the same secondary antibody. Following incubation at room temperature for 1 hour, the membranes were washed four times at 10 min intervals with 2nd wash buffer (620mM NaCl/1st wash buffer) and the final wash drained off. In a separate dry container, equal volumes of "detection reagent 1" and "detection reagent 2" were mixed and the membranes immersed in the mix with shaking for 60 seconds. The detection reagents were then decanted off, membranes were wrapped in cling-film and exposed to Kodak film. Finally, the films were developed using the X-OMAT processor.

FIGURE 2.1

IMMUNODETECTION USING ECL



The principles of ECL Western blotting as described in Amersham "ECL Western blotting protocols".

2.7 MOLECULAR BIOLOGY

2.7.1 'Mini preparation" of plasmid DNA

2ml of 2xYT containing 25µg/ml of ampicillin (Sigma, Chem. Co.) was innoculated with a single putatively transformed bacterial colony, and the culture was shaken overnight at 37°C. Plasmid DNA was isolated from other bacterial contaminants by the following procedure in which all steps were performed on ice.

Cells were pelleted in 1.5ml microfuge tubes, the supernatant discarded and the pellet resuspended in 100µl of glucose buffer (see Table 2.1). After incubating on ice for 5 min, bacterial lysis was completed by the addition of 200µl of alkaline lysis buffer (see Table 2.1). The samples were shaken gently and incubated on ice for 10-20 min to precipitate high molecular weight DNA. Precipitation of dissolved protein/SDS was obtained by the addition of 300µl potassium acetate, pH 5.5 (see Table 2.1) to the sample, incubating on ice for 5 min and microfuging at maximum speed for 5 min.

Phenol extraction was then carried out to de-proteinise the nucleic acids :- one phenol extraction, one phenol/chloroform (1:1 (v/v) extraction and two chloroform: isoamyl alcohol (24:1(v/v)) washes were carried out on the upper aqueous phase, produced after each previous treatment. 275µl of 3M sodium acetate, pH 5.5 followed by 1.5ml of ice cold ethanol was added to each sample and, after incubating at -20°C for 1-24 h, the precipitated plasmid DNA was pelleted by microfuging at maximum speed for 30 min The resulting pellets were then washed with 70% ethanol, microfuged for 15 min and placed in a dessicator until dry. Finally, the pellets were resuspended in 20µl of sterile H_20 and the DNA concentration and purity was calculated after determining absorbance values at 280nm and 260nm.

TABLE 2.1

REAGENTS FOR MOLECULAR BIOLOGY

REAGENT	AMOUNT	CHEMICAL	Final volume with H_2O (ml)
2xYT	16g	Bactotryptone	
	10g	Yeast extract	1000
	5g	Sodium chloride	
2xYT AGAR	16g	Bactotryptone	
	10g	Yeast extract	1000
	5g	Sodium chloride	
	15g	Bactoagar	
Glucose buffer	4.5g	Glucose	
	12.5ml	1M Tris-HCl pH 7.4	500
	31.2ml	0.2M EDTA	
Potassium acetate buffer	60ml	5M Potassium acetate	100
	11.5ml	Glacial acetic acid	
TE buffer	0.121g	Tris-HCl pH 7.4	100
	0.037g	EDTA	
Alkaline lysis buffer	5ml	20% SDS	100
	2ml	10M NaOH	

2.7.2 Preparation of competent bacteria for transformation with plasmid DNA.

Bactotryptone, Yeast extract and Bactoagar for the preparation of bacterial media were supplied by DIFCO Laboratories, Detroit, U.S.A.

2ml of 2xYT medium was innoculated with a single colony of JM109 or XL-1 blue *Escherichia coli* (*E.coli*).The culture was incubated overnight with shaking at 37°C. The following day, 40ml of sterile 2xYT medium (contained in a conical flask) was innoculated with 2ml of the overnight culture. The flask was then incubated at 37°C with shaking until the cells had reached log phase in their growth cycle (log phase was achieved when the O.D. reading at 550nm was between 0.5 and 0.7 for a 1ml sample of the culture).

All subsequent steps were carried out on ice unless otherwise stated.

The culture was then transferred to a pre-chilled 50ml polyethylene centrifuge tube and centrifuged at 6000rpm for 3 min at 4°C (J2-21 rotor). The supernatant was discarded and the pellet gently resuspended in 20ml of ice cold 50mM $CaCl_2$. After incubating on ice for 20 min, the culture was centrifuged at 4350 x g for 3 min 4°C and the pellet resuspended in 4ml of ice cold 50mM $CaCl_2$. The cells were incubated on ice for at least a further 60 min prior to transformation.

Finally, sterile glycerol was added to 15% (v/v), the samples were aliquoted, snap frozen in liquid nitrogen, and stored at -80°C until required.

2.7.3 Transformation of competent bacteria with plasmid DNA.

200µl of competent *E. coli* were thawed on ice and then mixed with 1-10µl of plasmid DNA (0.3-0.5µg) in a pre-chilled polyethylene 5ml bijou bottle. The mix was incubated on ice for 90 min and then gently shaken for 2 min in a water bath at 37°C to heat shock the cells. The mix was then quickly placed on ice and 0.5ml of

2xYT medium was added. The mixture was shaken at 37°C for 30-60 min and then spread gently over 2xYT agar plates containing 25μg/ml ampicillin. The plates were incubated overnight at 37°C and the following morning a number of single colonies were selected and grown in 3ml of 2xYT medium containing 25μg/ml ampicillin. The plasmid DNA was then isolated (see 2.7.1), and characterised by restriction digestion (see 2.7.6).

2.7.4 Large scale preparation of plasmid DNA

500ml of 2xYT was innoculated with a small volume of culture prepared from transformed *E. coli* and the culture shaken overnight at 37°C. The following morning, cells were harvested at room temperature by centrifugation in Beckman 200ml polypropylene bottles at 3840 x g for 5 min at 4°C (JA 14 rotor).

The pellets were then resuspended in 9ml total volume of ice-cold glucose buffer (see Table 2.1) and transferred to 50ml Beckman polypropylene tubes. 0.5ml of 50μg/ml lysozyme solution was then added to lyse the cells, and the mix was incubated at room temperature for 5 min. Following this, 10ml of alkaline lysis buffer (see Table 2.1) was added and the mix again incubated for 5 min at room temperature. Finally 7.5 ml of 5M potassium acetate buffer, pH 5.5 was added, the tubes shaken vigourously and then incubated on ice for at least 10 min when the solution appeared thick and viscous. After centrifugation at 48000 x g for 20 min at 4°C (JA 20 rotor), the supernatants were strained through 2 layers of muslin into new polypropylene 50ml centrifuge tubes, mixed with an equal volume of propan-2-ol and incubated at room temperature for 30-60 min. After centrifuging at 17400 x g for 30 min at room temperature (JA 20), the supernatant was discarded and pellets were washed, without resuspension, with 20ml of 70% ethanol. The ethanol was then carefully decanted and the pellet dried in a dessicator until "just dry". The pellets were then pooled and resuspended in a total volume of 30ml of TE buffer (10mM Tris, 1mM EDTA, pH 8.0). This solution was then transferred to two 50ml

polyethylene tissue culture tubes, each containing 30g of caesium chloride, and the contents were dissolved by shaking. 2.4ml of 10mg/ml ethidium bromide was added to the contents of each tube, the solutions carefully transferred to Beckman VTi50 tubes and the tubes were heat-sealed. After centrifugation at 226000 x g (VTi50 rotor) for 20h at 20°C, the tubes were viewed under U.V. light. A number of phases were present, but the DNA was clearly visible as a tight fluorescent band located midway up the tube. (The protein was present at the surface while the RNA formed the pellet).

The DNA band was removed with a syringe and 18.5 gauge needle and the red-coloured ethidium bromide was extracted by adding an equal volume of butan-1-ol. After incubating at room temperature for 5 min the mix separated into 2 phases and the red-coloured upper phase, containing ethidium bromide, was discarded. The ethidium bromide extraction procedure was repeated a further 3 times until the lower phase was completely clear in colour. This aqueous phase was then transferred to dialysis tubing (SPECTRA/POR, Spectrum Medical Industries, INC.) and dialysed against 2 litres of TE buffer for 4 hours at 4°C.

After this time, the contents of the dialysis tubing were pooled, transferred to 50ml polypropylene tubes and 2.5 volumes of ethanol added. After incubating at -20°C overnight, DNA was pelleted by centrifuging at 10400 x g for 30 min at 4°C (JA 20 rotor). Finally, the pellet was dried and resuspended in 0.5ml of TE buffer. The DNA concentrations and purity were determined by reading the absorbance at 260 and 280 nm.

2.7.5 Calculation to determine the plasmid DNA concentration and purity

absorbance value of 1.0 at 260nm = 50μg of double-standed DNA

absorbance value of "y" at 260nm = "y" X 50μg of double stranded DNA

absorbance at 260nm

_____ = " Z " DNA has a lower protein

absorbance at 280nm concentration and has a higher purity

when the value of" Z " is nearer to 2.0.

2.7.6 Restriction enzyme digestion of DNA.

All molecular biology enzymes and reagents were supplied by Promega, Southampton, UK unless stated otherwise.

Approximately 0.5μg of DNA was digested in a reaction volume of 20μl, and digestion was carried out at 37°C for 90 min.

0.5-1.0μg of DNA, 2μl of 10X restriction buffer and 0.5-1.0 units of restriction enzyme were added to a sterile 1.5ml microfuge tube and the volume made up to 20μl with distilled H_2O. 1μl of 1mg/ml RNase was added after 60 min to digest any RNA present and after a further 30 min 5μl of 5X DNA loading buffer (0.25% bromophenol blue, 30% (v/v) glycerol/H_2O) was added to terminate the reaction. The restriction digest mix was then vortexed and the total volume of sample was electrophoresed on a 1% agarose gel in a buffer tank filled with 1X TAE buffer containing 25μg/ml ethidium bromide. Electrophoresis was carried out until the dye

front had migrated two thirds along the gel and the DNA fragments were viewed under U.V. light. A sample of *Bst E* II-digested λ DNA was also included on the gel so the sizes of digested DNA fragments could be determined.

2.7.7 Agarose gel electrophoresis

For standard 1% agarose gels, 0.5g of agarose was dissolved by boiling in 50ml of distilled water and cooled to hand hot temperature and then 1ml of 50X TAE (2mM Tris, 50mM EDTA, 5.7% (v/v) glacial acetic acid) was added. The gel solution was poured into a 50ml horizontal electrophoresis gel cartridge (BRL model H5), with the comb already in place, and left to set at room temperature for 15 min. The cartridge was transferred to the electrophoresis tank containing 1X TAE buffer (0.04mM Tris, 1mM EDTA, 0.114% (v/v) glacial acetic acid) and the comb was carefully removed. The DNA samples were then loaded and 20μl of 10mg/ml ethidium bromide was added to the tank buffer. The samples were electrophoresed at 50-100mA using an LKB 2197 power supply until the dye front had migrated two thirds of the way along the gel towards the positive electrode. The DNA was visualised by U.V. light using an Ultraviolet Products Inc. transillumintor, and photographed using a Mitsubushi video copy processor.

2.7.8 De-phosphorylation of double-stranded DNA using calf intestinal phosphatase

Plasmid DNA was digested with the appropriate restriction enzyme for 60 min and then RNAse treated for 30 min at 37°C as previously described. The mix was then made up to a volume of 100µl with dephosphorylation buffer (1mM $ZnCl_2$, 1mM $MgCl_2$, 10mM Tris-HCl pH 8.8) and 1 unit of Calf intestinal phosphatase (CIP) was added. After incubation at 37°C for 60 min, plasmid DNA was isolated from the enzyme reagents by phenol extraction and precipitation as described previously (2.7.1), and DNA was resuspended in 20µl of H_2O. Dephosphorylated plasmid DNA was subsequently ligated to GLUT 2 and GLUT 4 cDNA fragments (see 4.2.3 and 6.2.3)

2.7.9 Ligation of double-stranded DNA

DNA fragments encoding GLUT 2 or GLUT 4 (see 4.2.3 and 6.2.3) were ligated to plasmid DNA by the following procedure. In order to increase the efficiency of ligation, the reaction was performed with a high GLUT DNA: plasmid DNA ratio. (10:1).

Plasmid DNA which had been CIP-treated (see 2.7.8) was added to GLUT 2 or GLUT 4 cDNA at a molar ratio of 1:10 and the mix incubated at 60°C for 5 min. After pulsing the samples were briefly in a microfuge, 3µl of 5X concentrated ligation buffer, 1µl of 10mM ATP and 2U of T4 ligase were added and the samples then incubated overnight at 6°C.

Competent bacteria were transformed with the ligation reaction (see 2.7.2) and then plasmid DNA was isolated from bacterial colonies (see 2.7.1). Finally, the DNA was characterised by restriction digestion and agarose gel electrophoresis as

described previously (see 2.7.6, 2.7.7), to verify that GLUT cDNA had ligated with plasmid DNA and that plasmid DNA had not self-ligated.

2.8 CELL CULTURE PROCEDURES

2.8.1 Cell culture media

All cell culture reagents:- Dulbecco's Modified Eagles medium (DMEM), bovine serum, penicillin/streptomycin, 0.5% lyophilised trypsin, and Geneticin (G-418-sulphate) were obtained from GIBCO Life Technologies LTD, Paisley, Scotland.

Dulbecco's Modified Eagles Media (DMEM) contained 4500mg/l glucose but no sodium pyruvate. All newborn calf serum and foetal calf serum was screened for mycoplasma and viruses.

Cell culture plasticware was obtained from NUNC and FALCON (Beckton Dickson) unless otherwise stated.

2.8.2 Cell culture growth media

3T3-L1 murine fibroblast cells

10% (v/v) Newborn calf serum/ DMEM containing 200 IU penicillin and 200µg streptomycin/500ml.

Transfected 3T3-L1 murine fibroblast cells

20% (v/v) Newborn calf serum/DMEM containing 200 IU penicillin and 200µg streptomycin/500ml.

H4IIe rat hepatoma cells

10% (v/v) Foetal calf serum/DMEM containing 200 IU penicillin and 200μg streptomycin/500ml.

2.8.3 Freeze media compositions

3T3-L1 murine fibroblast cells

90% (v/v) 20% calf serum/DMEM medium containing 10% (v/v) sterile glycerol, equilibrated at in an incubator 37°C with 10% CO_2 for 60 min.

H4IIe rat hepatoma cells

70% (v/v) 10% foetal calf serum/DMEM medium, supplemented with 20% (v/v) foetal calf serum, 10% (v/v) filter sterilised DMSO.

2.8.4 Preparation of 3T3-L1 fibroblast differentiation medium

Differentiation reagents, unless otherwise stated, were obtained from Sigma Chemical Company (Poole, Dorset, U.K).

Differentiation medium containing 10% foetal calf serum (v/v), 0.5mM methyl isobutylxanthine, 0.25μM dexamethasone, and insulin (1μg/ml) was prepared as outlined below.

2.5mM dexamethasone in ethanol was diluted 1:20 with 10% (v/v) foetal calf serum/DMEM medium immediately prior to use yielding a 500X stock solution. A 500X concentrated sterile solution of methyl isobutylxanthine (IBMX) was also prepared by dissolving 55.6g IBMX in 1.0ml of 0.35N KOH and passing the

solution through a 0.22 micron filter. Insulin (1mg/ml) was then prepared in 0.01N HCl, and filter sterilised by passing through a 0.22 micron filter.

3T3-L1 differentiation medium was prepared by diluting both the dexamethasone and IBMX solutions to a 1X concentration in 10% (v/v) foetal calf serum/DMEM and then adding insulin to a final concentration of 1μg/ml.

2.8.5 3T3-L1 fibroblast cell differentiation protocol

3T3-L1 fibroblast cells, grown on 10cm cell culture plates and 48-72h prior to confluence, were trypsinised and used to seed either ten to twelve 10cm plates or six to eight 6-well plates and four 10cm plates. The cells were maintained in 10% (v/v) newborn calf serum/DMEM until 48h post confluence, at which time growth medium was aspirated and replaced with differentiation medium consisting of 10% (v/v) foetal calf serum/DMEM containing 0.25μM dexamethasone, 0.5mM methyl isobutylxanthine and 1μg/ml insulin. After a further 2 days this medium was aspirated and replaced with 10% foetal calf serum/DMEM containing insulin at 1μg/ml. The cells were incubated in this medium for 2 days and then the medium was aspirated and replaced with 10% (v/v) fetal calf serum/DMEM. Cells were used 8-13 days post differentiation, at which time insulin-stimulated 2-deoxyglucose uptake was maximal (Frost and Lane, 1985).

2.8.6 Preparation of cells for freezing

DMEM medium was aspirated from 10cm cell culture plates and 5 ml of filter sterilised 0.05% (w/v) trypsin/PBS, pH 7.4 added to each plate. Plates were then incubated at 37°C for 3-5 min until the cells were just beginning to lift off the plate. The trypsin solution was gently titurated over the surface of the plate until all of the cells were detached. After transferring to a 30ml sterilin universal tube, the trypsin/cell mix was centrifuged at 1000 rpm for 5 min and the trypsin supernatant

was aspirated. The cell pellet was then resuspended in 1ml of freeze medium per 10cm plate trypsinised (see 2.8.3), the cells were aliquoted into 1.8ml polypropylene Cryo tubes and stored overnight at -80°C. The following morning the vials were transferred to liquid nitrogen and stored until required.

2.8.7 Resurrection of frozen cell stocks from liquid nitrogen

Cell vials were removed from liquid nitrogen and incubated in a water bath at 37°C until the cells were just thawed. Vials were then transferred to a cell culture sterile flow hood (MDH InterMed) where the following procedure was performed.

The cells were titurated gently using a sterile pasteur pipette to disperse any large aggregates of cells and transferred to culture plates containing the appropriate medium (see 2.8.2). Cells were then maintained in an incubator at 37°C in an atmosphere of 10% CO_2. The following day the medium was aspirated to remove cell debris and replaced with fresh growth medium.

2.8.8 Trypsinisation of cells

When cells were 70-80% confluent, DMEM growth medium was aspirated and 5 ml of sterile 0.05% (w/v) trypsin/PBS pH 7.4 was added to each 10 cm plate. Plates were then incubated at 37°C until the cells were beginning to lift off the plate and the trypsin was titurated over the surface of the plate until all of the cells were detached. After centrifuging the cell/trypsin mix at 1000 rpm for 5 min in a 30 ml Sterilin tube, the trypsin supernatant was aspirated and the cell pellet resuspended in a small volume of DMEM growth medium. This was then diluted in an appropriate volume of DMEM growth medium and used to seed ten to twelve 10cm cell culture plates.

2.8.9 Collagen coating of cell culture plasticware

During differentiation, it was observed that GLUT 2-transfected 3T3-L1 fibroblasts had a tendency to peel off from the plate surface. Collagen treatment of plates was therefore performed to promote better cell adhesion.

Collagen, derived from rat tail (Sigma), was dissolved in 3% acetic acid to a final concentration of 0.3mg/ml. Using a syringe and needle, the collagen solution was applied to the plate briefly and then removed to leave a thin film coating. Plates were then dried in a sterile flow hood and irradiated with U.V. light overnight. Before use, plates were washed with 5 ml serum-free DMEM medium to ensure that all traces of acetic acid had been eradicated.

2.8.10 Transfection procedure using DOTAP (-N-[1-(2,3-Dideolyloxy)propyl]-N,N,N-trimethylammoniummethylsulphate)

The reagent composition outlined below was sufficient for the transfection of one 10cm plate of cells.

70µl of the transfection reagent DOTAP (1mg/ml) was diluted to a volume of 250µl (Boehringer Mannheim Biochemica) with sterile HBS (20mM HEPES, 150mM NaCl pH 7.4) in a 10ml polypropylene tube. In a separate polypropylene tube, 5µg of DNA was diluted to 250µl with HBS and, following this, the DNA and DOTAP solutions were mixed and left to incubate at room temperature for 10 min. Meanwhile, DMEM medium was aspirated from cells to be transfected and the cells were washed twice with 5ml of serum-free DMEM medium.

Following the addition of 5ml of serum-free DMEM medium to the cells, the transfection reagent-DNA mixture was added dropwise using a sterile glass pasteur pipette. The contents of the culture dish were swirled gently between drops to ensure even distribution of the mix, and the cells were then incubated at 37°C in an

atmosphere of 10% CO_2 for 24h. Finally, the medium was aspirated and replaced with DMEM medium containing the appropriate serum (2.8.2).

2.8.11 Sub-cloning transfected cells

2-3 weeks after transfection and antibiotic selection, discrete colonies of cells became apparent. A number of these antibiotic-resistant colonies were isolated by the following protocol, expanded in antibiotic-containing medium and screened by immunoblotting for the expression of the transfected DNA.

The growth medium was aspirated and 2-3ml of sterile 0.05% trypsin/PBS, pH 7.4 was added to each 10 cm plate. Plates were incubated at room temperature for 2 min and then, using a sterile yellow-tipped pipetteman, the colony was gently scraped from the plate and transferred, together with the surrounding trypsin, to a 6cm plate containing DMEM growth medium. The cells were then maintained in an incubator at 37°C with 10% CO_2. After 2-3 days, 2ml of the growth medium was drawn off and replaced with 2ml of antibiotic-containing growth medium and this procedure was repeated every two days until the cells were 80% confluent. Cells were then trypsinised, and used to seed four to six 10cm plates. These cells were grown in antibiotic-containing growth medium until 80% confluent, after which time cells were frozen or used to prepare lysate samples for immunoblotting.

2.9 MEMBRANE PREPARATION PROCEDURES

2.9.1 Sucrose gradient preparation of liver cell membranes

Purified plasma membranes were prepared from total rat liver homogenates as described by Pilkis *et al.* (1974) and as modified by Marchmont *et al.* (1981).

3 male Sprague-Dawley rats (220-250g) were stunned and killed, the livers were carefully excised and rinsed with ice-cold potassium carbonate buffer (1mM $KHCO_3$ pH 7.2). Livers were then chopped finely using scissors and homogenised with 6 strokes using a Tri-R homogeniser (model K-34) at speed 3 in 3 volumes of ice-cold $KHCO_3$. The homogenate was filtered through 2 layers of muslin, the volume made up to 300ml with ice cold $KHCO_3$ and divided between six 50ml Nalgene Oak Ridge centrifuge tubes. After centrifuging at 2000 x g for 10 min at 4°C (MSE21, 8x50 rotor), the supernatant was discarded. The pellets were poured into a pre-chilled polythene beaker on ice, containing 78g of sucrose, and the volume made up to 130ml with $KHCO_3$ buffer. After mixing on ice for 30 min, 20ml aliquots of the homogenate were then layered onto a sucrose gradient comprising 6.5 ml 42.5% sucrose (w/v)/3mM imidazole pH 7.4 and 12 ml 48.5% sucrose (w/v)/3mM imidazole, pH 7.4. Following centrifugation at 133,000 x g for 3 hrs 10 min at 4°C (27.2 rpm, SW28 rotor), a number of phases were present (see Fig 2.2).

The "fluffy brown" layer, containing plasma membranes, was carefully removed with a pasteur pipette, diluted with an equal volume of $KHCO_3$ buffer. This was transferred to a pre-chilled Nalgene Oak Ridge centrifuge tube and centrifuged at 25,000 x g for 15 min at 4°C (14.4 rpm MSE21, 8 X 50). Finally, the supernatant was discarded, the pellet was resuspended in 5ml of ice cold $KHCO_3$ and the membranes were stored as 100µl aliquots at -80°C until required.

2.9.2 3T3-L1 adipocyte subcellular fractionation

Four fractions, similar to those described for the fractionation of rat adipocytes, were obtained according to the method of Piper *et al.* (1991)

3T3-L1 adipocytes were incubated at 37°C in serum-free DMEM medium for 2-3 hours prior to membrane preparation and then for 30 min in the presence or absence of 1µM insulin. Stimulation was terminated by aspiration of the incubation medium and the cells were washed 3 times with 5ml of ice cold HES (255mM

FIGURE 2.2

Sucrose Gradient Preparation of Liver Plasma membranes

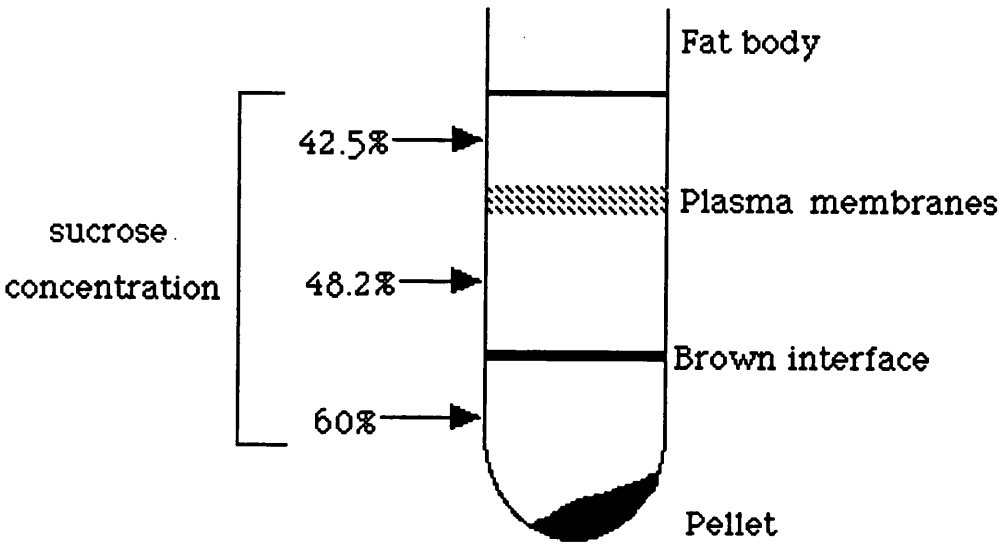
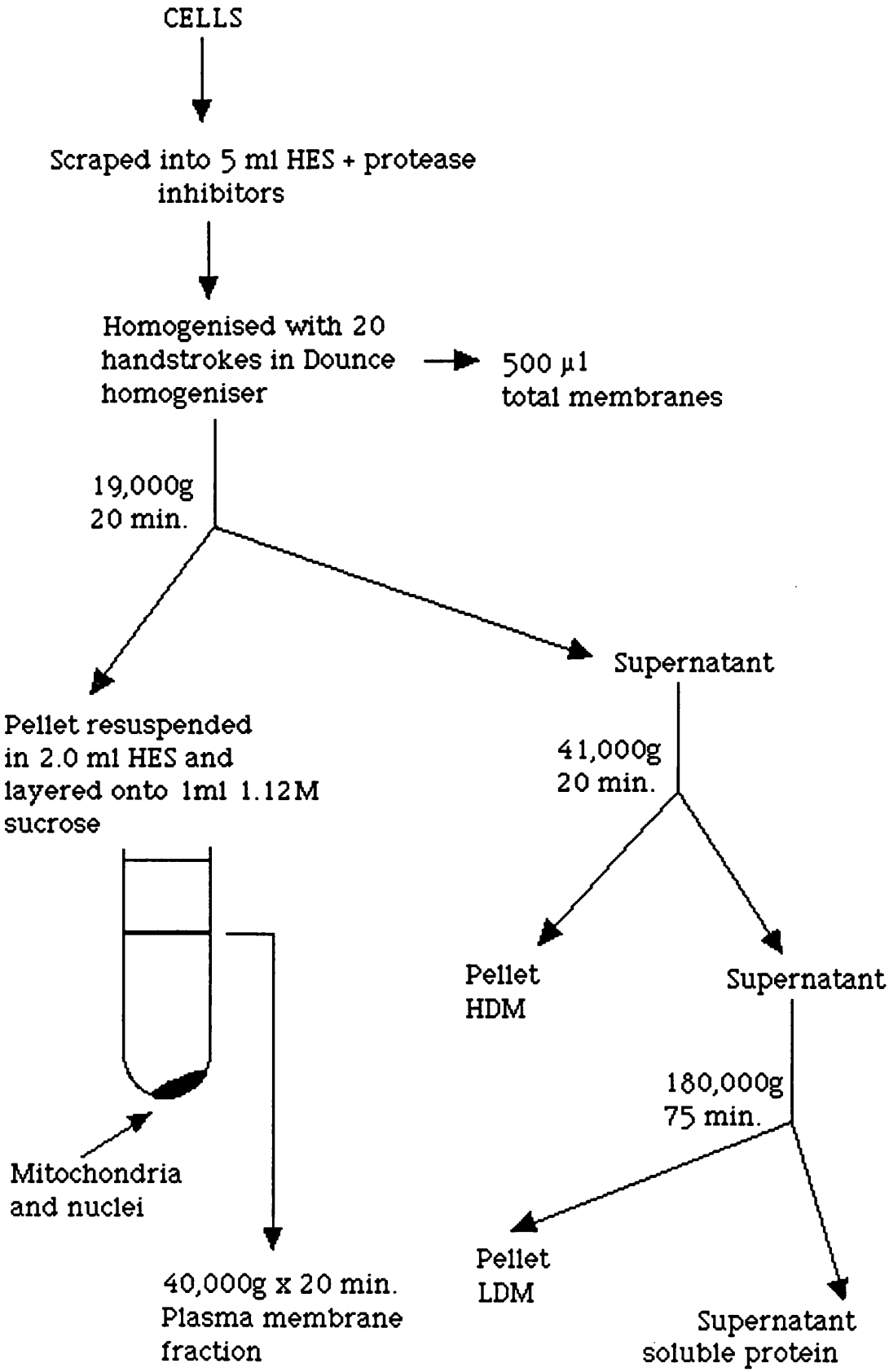


FIGURE 2.3

SUBCELLULAR FRACTIONATION OF 3T3-L1 ADIPOCYTES



sucrose, 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.4 and 1mM EDTA). All subsequent steps were performed at 0-4°C.

Cells were scraped into 5ml of ice cold HES containing 1µg pepstatin A, 0.2mM DFP and 4µM Ep64 protease inhibitors, and cells were homogenised with 20 hand strokes in a loose-fitting Dounce homogeniser. At this stage in the procedure a 500µl aliquot of the homogenate was retained and designated as a total membrane fraction. Homogenates were transferred to pre-chilled Oakridge Beckman centrifuge tubes and then centrifuged at 19,000 x g for 20 min at 4°C (JA 20 rotor).

The resulting supernatant was centrifuged at 41,000 x g for 20 min (JA 20 rotor), yielding a pellet designated as the high-density microsomal fraction (HDM). The supernatant from this spin was centrifuged at 180,000 x g for 75 min at 4°C, yielding a pellet designated as the low density microsomes (LDM).

Meanwhile the pellet from the initial 19,000 x g spin was resuspended in 2ml ice-cold HES, homogenised with 5-10 handstrokes in a loose-fitting Dounce homogeniser and layered onto 10ml of 1.12mM sucrose in HES, in a 5ml Beckman Ultra-clear tube (344062 11 x 60mm). After centrifuging at 100,000 x g for 60 min at 4°C in a SW 60 swing out rotor, a brown fluffy band at the interface (plasma membrane fraction) and a viscous brown pellet (mitochondria/nuclei fraction) were obtained. The plasma membrane fraction was resuspended in 10ml of HES and pelleted at 40,000 x g for 20 min at 4°C. All fractions were resuspended in HES at a final concentration of 1-5 mg/ml, snap frozen in liquid nitrogen and stored at -80°C.

2.10 Glucose uptake assay

Hexose uptake was measured by the uptake of 2-deoxyglucose according to the method of Gibbs *et al.* (1988) and Frost and Lane (1985).

Cells were cultured on 6-well plates and incubated in serum-free Dulbecco's Modified Eagles medium for 2 h prior to use. Cells were washed 3 times with 3ml of KRP buffer (64mM NaCl, 2.5mM KCl, 2.5mM $NaH_2PO_4.2H_2O$, 0.6mM

$MgSO_4.7H_2O$, 0.6mM $CaCl_2$, pH 7.4) and then incubated at 37°C in 950µl of KRP with or without 1µM insulin for various stimulation times. Hexose uptake was initiated by the the addition of 50µl of [^{3}H] 2-deoxyglucose, such that the final concentrations were 100µM and 0.5µCi/ml.

After 5 min, the reaction was terminated by inverting the plates rapidly to remove the incubation buffer and and then immersing them sequentially in 3 volumes of ice-cold PBS, pH 7.4. The plates were left to air-dry, 1ml of 1% Triton X-100/H_2O was added to cell monolayers and [^{3}H] 2-deoxyglucose uptake was determined by liquid scintillation counting.

2.11 Deglycosylation of cell surface proteins

100-150µg of rat liver, mouse liver, mouse brain or 3T3-L1 adipocyte membranes were resuspended to a volume of 500µl in de-glycosylation buffer (50mM potassium phosphate pH 5.0, 20mM EDTA, 2% Triton X-100, 0.2% SDS, 1% β 2-mercaptoethanol), and incubated at 37°C for 48h with 0.35 U of N-glycosidase F and 5µg of neuraminidase (Boehringer Mannheim GmbH). Samples were briefly vortexed at intervals throughout the incubation and proteins were then precipitated after the addition of 50lµ of 50% trichloroacetic acid. After the samples were microfuged at maximum speed for 4 min, protein pellets were resuspended in 1X SDS-PAGE sample buffer (93mM Tris-Cl pH 6.8, 2% SDS, 10% (v/v) glycerol, 10mM Na_2EDTA, 0.007% bromophenol blue), electrophoresed on 10% polyacrylamide gels and proteins transferred to nitrocelluose membranes. The nitrocellulose membranes were then immunoblotted with specific anti-GLUT antibodies.

CHAPTER 3

THE EXPRESSION AND DISTRIBUTION OF THE BRAIN-TYPE GLUCOSE TRANSPORTER, GLUT 3, IN MURINE TISSUES

3.1 INTRODUCTION

Meeting the energy requirements for oxidative metabolism in the mammalian brain is almost exclusively dependent on a sustained supply of glucose (Owen *et al.*, 1967; Lund-Anderson, 1979). Of the 6 major isoforms cloned, GLUT 1 has been described to constitute the blood-brain barrier glucose transporter, mediating the transport of glucose from the circulation into the brain interstitium (Froehner *et al.*, 1988). However, RNA blotting studies have shown that in addition to GLUT 1, the GLUT 3 isoform is also expressed in the adult human, rabbit, monkey, rat, mouse (Yano *et al.*,1991) and dog brain (Gerhart *et al.*, 1992). It is therefore possible that this protein is responsible for the transport of glucose across the plasma membrane of neuronal and glial cells. GLUT 3 was first cloned from human fetal skeletal muscle (Kayano *et al.*, 1988), but recently GLUT 3 homologues have also been identified and cloned from mouse (Nagamatsu *et al.*, 1992) and chicken (White *et al.*, 1991). The human and mouse GLUT 3 proteins are highly homologous, having 83% amino acid identity however, substantial differences exist in the extracellular loop connecting transmembrane regions 1 and 2 and at the intracellular COOH-terminal domains (Nagamatsu *et al.*, 1992).

Kinetic studies have recently shown that GLUT 3 and GLUT 1 isoforms, when expressed in *Xenopus laevis* oocytes, exhibit K_ms for 3-O-methyl glucose of 10.6mmol/l and 17mmol/l respectively (Gould *et al.*, 1991). The low K_m of GLUT 3 may represent an adaptation to the lower glucose concentrations which exist in the extracellular fluid of the brain as compared to the blood plasma. The expression of

GLUT 3 would thus ensure efficient glucose uptake when glucose levels in the blood plasma were low.

Northern blot analysis of GLUT 3 expression in human tissues has shown that GLUT 3 is expressed at highest levels in the brain, although its mRNA has also been detected in adipose tissue, liver and muscle, all of which are insulin-sensitive. On this observation it has been suggested that this isoform could also be important in regulating glucose disposal in those tissues in response to insulin.

In one study, Western blot analysis of human tissues, utilising affinity purified antibodies raised to the COOH-terminus of human GLUT 3, indicated that GLUT 3 protein was detectable in the human brain as a 45-48 kDa protein. Lower levels of the protein were just detectable in the heart, placenta and liver but not in muscle and fat tissues (Shepherd *et al.*, 1992).

RNA blotting studies in monkey, rabbit, and mice have shown that GLUT 3 mRNA has a more restricted tissue distribution in these species compared to humans and that it is expressed primarily in brain (Yano *et al.*, 1991). However, these studies did not examine the pattern of expression of GLUT 3 in the brain or show the presence of GLUT 3 protein.

Studies of peripheral insulin resistance routinely utilise rodent models of diabetes and/or obesity such as the db/db mouse or BB rat (Coleman, 1978; Tominaga *et al.*,1986), since these tissues are easy to obtain and prepare compared to their human counterparts. Therefore, as a consequence of the availability of murine tissue, an anti-peptide antibody against the COOH-terminal 14 amino acids of murine GLUT 3 was generated. This antibody was subsequently used to determine the sites of expression of GLUT 3 protein in membranes prepared from mouse liver, muscle and from murine 3T3-L1 adipocyte cells. This study was undertaken to determine the tissue distribution of GLUT 3 protein and to speculate on its potential role in the context of whole body glucose homeostasis.

3.2 METHODS

3.2.1 GLUT 3 antisera production

A peptide corresponding to the COOH-terminal 14 amino acids of the murine homologue of GLUT 3, sequence NH_2-LNSMQPVKETPGNA-COOH was coupled to keyhole limpet haemocyanin via an additional N-terminal cysteine residue and used to generate anti-GLUT 3 peptide antibodies in rabbits (see 2.1.1). The antiserum was then purified by antigen-antibody immobilisation affinity purification (see 2.1.2).

Affinity purified anti-GLUT 1 antibodies were a gift from Dr. S. A. Baldwin, Royal Free Hospital, London, U.K.

3.2.2 Mouse brain membrane preparation

Intact mouse brain tissue was dissected from two mice and homogenised by 20 strokes in a Dounce homogeniser in 10ml of Tris-EDTA buffer (10mM Tris-HCl pH 7.5, 1mM EDTA) containing protease inhibitors (1μg pepstatin A, 0.2mM DFP and 4μM Ep 64). The homogenate was centrifuged at 2,500 x g for 10 min at 4°C, and the supernatant was collected. This supernatant was then further centrifuged at 25,000 x g for 10 min at 4°C to pellet total membranes. The resulting pellet was washed in Tris-EDTA buffer, centrifuged at 25,000 x g for 10 min at 4°C, was then resuspended in 5ml of Tris-EDTA buffer, and frozen at -80°C prior to use.

3.2.3 Liver plasma membrane and 3T3-L1 adipocyte membrane preparations

Liver plasma membranes, 3T3-L1 adipocyte plasma membrane and low density microsomal membranes were prepared as described previously (see 2.9.1 and 2.9.2).

3.2.4 Skeletal soleus muscle membrane preparation

Skeletal muscle membranes were prepared by Dr Barbara Kahn at Beth Israel Hospital Boston, according to the method of Klip and Marette (1992).

3.2.5 Neuroblastoma x glioma NG 108 membrane preparation

These hybrid cell membranes were prepared according to the method of Hamprecht *et al.* (1985), and were a gift from Dr G. Milligan, Department of Biochemistry, Glasgow University.

3.3 RESULTS

3.3.1 Characterisation of GLUT 3 anti-peptide antibody

Immunoblotting of brain membranes from mouse tissue with affinity-purified anti-GLUT 3 antibodies (Fig.3.1a) identified a major band at 50 kilodaltons, but prolonged exposure of the autoradiogram showed that fainter bands were also detectable at 55, 66, and 76 kilodaltons.

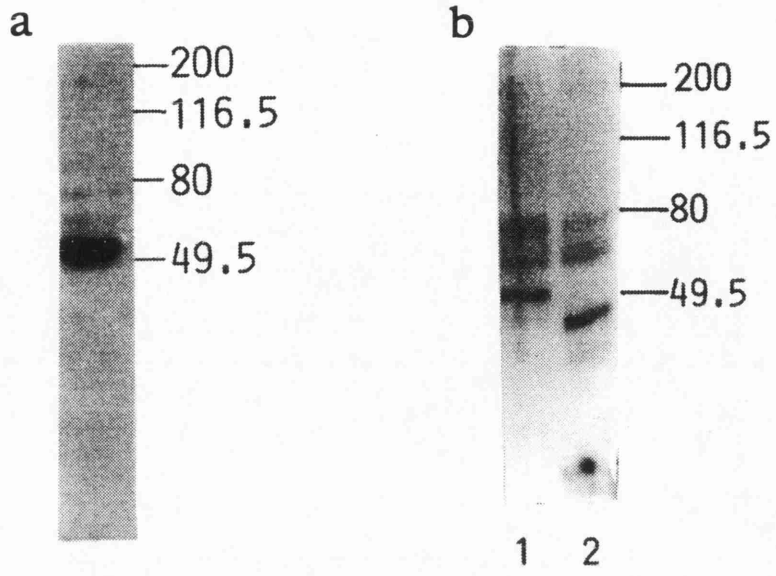
The ability of purified anti-GLUT 3 antibodies to recognise GLUT 3 protein has been demonstrated by cytochalasin B binding of immunoprecipitated GLUT 3 from mouse brain membranes (Gould *et al.*, unpublished data). It was observed in this study that cytochalasin B binding was blocked by 250mM D-glucose but not by 250mM L-glucose. These results thus indicated that the immunoprecipitated protein was a glucose transporter of the facilitative-diffusion type. Moreover, it was shown by Gould *et al.* that, when the immunoprecipitated protein was immunoblotted, the GLUT 3 protein had an apparent molecular weight of 50kDa (Gould *et al.*, unpublished data).

This suggested that the fainter bands detectable at 55, 66, and 76 kDa, when mouse brain membranes were immunoblotted with purified anti-GLUT 3 antibodies, were non-specific cross-reacting species and did not represent modified forms of the protein. To demonstrate this further, a series of peptide competition experiments were performed.

3.3.2 Immunoblotting of membrane samples using anti-GLUT 3 and anti-GLUT 1 antisera pre-incubated with GLUT 3 or GLUT 1 peptide.

GLUT 1 and GLUT 3 antisera, pre-incubated in the presence or absence of peptides corresponding to the COOH-terminal 14 amino acids of GLUT1 or GLUT

FIGURE 3.1
Immunological identification of GLUT 3 in mouse brain.



Panel a: Immunological identification of GLUT 3 in mouse brain. 12μg of mouse brain membranes were immunoblotted as described previously and the blot was probed with 10μg/ml affinity purified anti-GLUT 3 antibodies. The relative positions of the molecular weight markers in kilodaltons are shown to the right of the gel and the entire running gel is shown.

Panel b: Effect of glycosidase treatment on GLUT 3. 115μg of mouse brain membranes were incubated in the presence (lane 2) or the absence (lane 1) of neuraminidase and endoglycosidase F as described in section 2.11. Samples containing equal amounts (10μg) of membrane protein were then electrophoresed and immunoblotted with 10μg/ml affinity purified anti-GLUT 3 antibodies as described previously (2.3, 2.4, 2.5.1, 2.6.1).

3, were used for immunoblotting to demonstrate that GLUT 1 and GLUT 3 anti-peptide antibodies were highly specific for their respective glucose transporter isoforms. 1mg/ml stock solutions of GLUT 3 peptide (NH_2-NSMQPVKETPGNA-COOH) and GLUT 1 peptide (NH_2-PEELFHPLGADSQV-COOH) were prepared in PBS, pH 7.4 at room temperature and added to GLUT1 or GLUT 3 antisera at a final concentration of 5μg/ml.

10-30μg of mouse brain membranes were electrophoresed on 10% SDS/polyacrylamide gels, proteins were transferred to nitrocellulose membranes and non-specific sites were blocked on the membranes as described previously (see 2.5.1 and 2.5.2). Nitrocellulose membranes to be probed with anti-GLUT 3 antisera were then transferred to 1% (w/v) non-fat milk/1st wash buffer containing 10μg/ml anti-GLUT3 antibody with 5μg/ml GLUT1 peptide or 5μg/ml GLUT 3 peptide. Meanwhile, membranes to be probed with anti-GLUT 1 antibodies were transferred to 3% (w/v) BSA in 1st wash buffer containing 10μg/ml anti-GLUT 1 antibody with 5μg/ml GLUT 1 peptide or 5μg/ml GLUT 3 peptide.

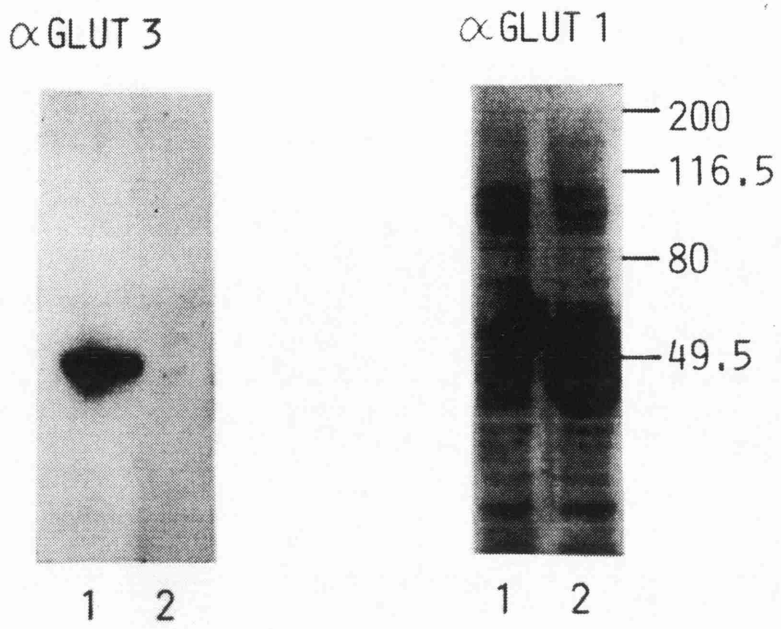
It was shown that for nitrocellulose membranes probed with anti-GLUT 3 antibodies, the major immunoreactive band in mouse brain membranes at 50kDa was effectively competed out when 5μg/ml of GLUT 3 peptide was present in the antiserum (Fig. 3.2a, lane 2) but not when 5μg/ml of the GLUT 1 peptide was present (Fig. 3.2a, lane 1). The higher M_r bands at 55, 66, and 76kDa were still present however when membranes were probed with GLUT 3 antibodies in the presence of GLUT 3 peptide.

These results indicate that the immunoreactive protein at 50kDa recognised by the anti-GLUT 3 antibodies is indeed mouse GLUT 3 and that the bands detected at higher molecular weights by anti-GLUT3 antibodies are non-specific cross-reacting proteins.

When nitrocellulose membranes were probed with anti-GLUT 1 antibodies, the GLUT 1 signal was not affected by the inclusion of 5μg/ml GLUT 3 peptide but was completely obliterated with the inclusion of 5μg/ml GLUT 1 peptide (Fig 3.2b).

FIGURE 3.2

Peptide competition analysis of GLUT 3 antibody.



10μg of mouse brain membranes were electrophoresed and transferred to nitrocellulose membranes as described previously (2.3 and 2.4). Nitrocellulose membranes were then probed with either anti- GLUT 3 or anti-GLUT 1 antibodies, as indicated. Included in the incubation buffer, containing the antibody, was 5μg/ml of GLUT 3 peptide (lane 2 in each case) or 5μg/ml of GLUT 1 peptide (lane 1 in each case). The entire running gel is shown and the positions of the molecular weight markers in kilodaltons are shown to the right of the gel.

3.3.3 De-glycosylation of GLUT 3

The predicted amino acid sequence of the GLUT 3 contains a potential site for N-linked glycosylation in the extracellular loop located between putative transmembrane helices 1 and 2 (Gould and Bell, 1990; Kayano *et al.*, 1990). The effect of glycosidases on the electrophoretic mobility of GLUT 3 was therefore examined in order to determine the presence and indeed the extent of glycosylation.

115μg of mouse brain membranes were solubilised and incubated in the presence and absence of 0.35U N-glycosidase F and 5μg neuraminidase as previously described (see 2.11). Samples, containing 10μg of membrane protein, were then electrophoresed on 10% SDS/polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting of membranes with anti-GLUT 3 antibodies showed that the apparent molecular weight of GLUT 3 had decreased from 50kDa to 43kDa (Fig. 3.1b, lane 2) in N-glycosidase F and neuraminidase -treated samples, but that the molecular weight was unaffected in membranes which were untreated (Fig. 3.1b lane 1). This decrease in molecular weight was consistent with the removal of a carbohydrate from the core polypeptide.

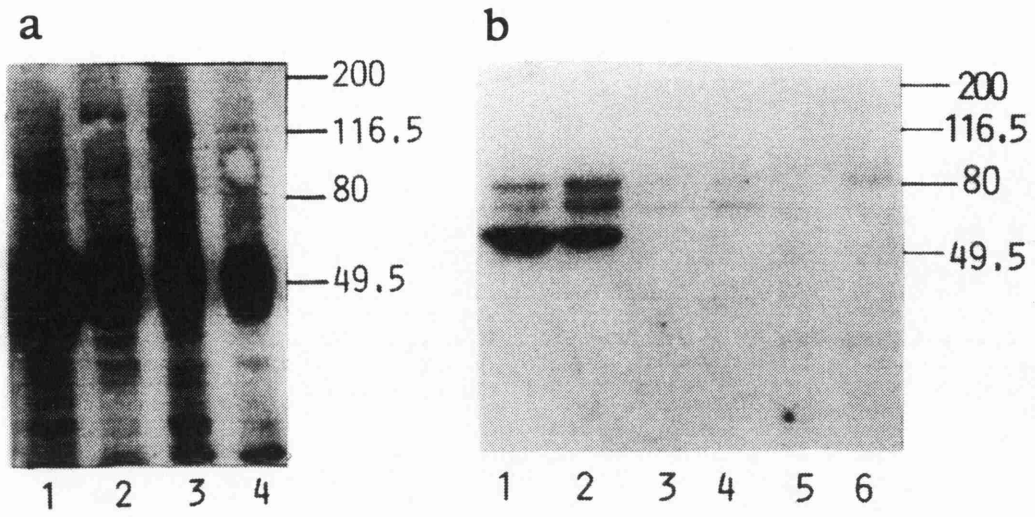
3.3.4 GLUT 3 tissue distribution

The distribution of GLUT 3 was examined in mouse liver, 3T3-L1 adipocytes and mouse muscle membranes by immunoblotting with anti-peptide GLUT 3 antibodies.

Subcellular fractions were prepared from 3T3-L1 adipocyte cells which had been incubated in the presence and absence of 1μM insulin (see 2.9.2). No immunoreactivity at 50 kilodaltons was observed in crude plasma membranes or in low density microsomal fractions prepared from insulin stimulated or basal cells (Fig. 3.3a, lanes 3, 4, 5, and 6). However GLUT 1, with an apparent molecular weight of

FIGURE 3.3

Tissue distribution of GLUT 3 and GLUT 1 in 3T3-L1 adipocyte membranes, mouse liver and mouse brain membranes



Panel a: Immunoblot analysis of liver, brain and 3T3-L1 adipocyte membranes with anti-GLUT 1. Samples contain 25μg of plasma membrane (lane 1) low density microsomes (lane 2) of 3T3-L1 adipocytes, 30μg of mouse liver plasma membranes (lane 3), or 10μg of mouse brain membranes (lane 4). These samples were immonoblotted with affinity purified anti-GLUT 1 antibodies.

Panel b: Tissue distribution of GLUT 3. 3T3-L1 adipocyte membranes fractions were prepared as described previously (2.9.2). Samples contain plasma membranes (lanes 3 and 4) and low density microsomes (lanes 5 and 6) prepared from basal (lanes 3 and 5) and insulin-stimulated (lanes 4 and 6) 3T3-L1 adipocytes. These fractions were blotted (25μg each lane) with affinity purified anti- GLUT 3 antibodies and the signals obtained were compared to those obtained from mouse brain membranes (lanes 1 and 2 ; 10 and 20μg respectively).

50kDa, was readily detectable in similar amounts of both plasma membrane and low density microsomal fractions (Fig. 3.3b, lanes 1 and 2).

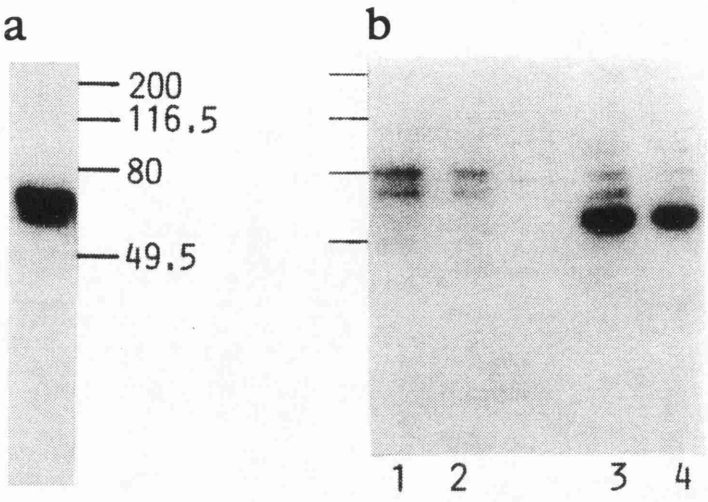
GLUT 2 immunoreactivity was observed at 59 kDa when 5μg of mouse liver membranes were probed with affinity-purified anti-GLUT 2 antibodies (Fig. 3.4a). GLUT 3 immunoreactivity at 50kDa was observed in both 10μg and 30μg of mouse brain membranes but not in either 15μg or 30μg of mouse liver membranes (Fig. 3.4b).

Skeletal muscle, being an important tissue for peripheral glucose disposal, was also examined for the expression of GLUT 3 protein. Membranes were prepared from mouse soleus muscle and immunoblotted with anti-GLUT 3 antibodies. The results indicated that, as expected, GLUT 4, the insulin-responsive transporter, and GLUT 1 were expressed but that there was no detectable GLUT 3 expression in either 50 or 100μg of membrane sample (Fig. 3.5).

The neuroblastoma x glioma cell line NG 108, a mouse x rat hybrid cell line, was also probed for the expression of GLUT 3 since the above results suggested that the expression of GLUT 3 might be restricted to brain and neuronal tissue. Immunoblot analysis of this cell line indicated that a band of 50kDa was present in 25μg of hybrid cell membranes but not in 25μg of membranes prepared from the parent rat glioma cell (Fig 3.6a). The expression of GLUT 1 was readily detectable in identical amounts of membranes from both samples (Fig. 3.6b).

FIGURE 3.4

Tissue distribution of GLUT 3 and GLUT 2 in mouse liver plasma membranes

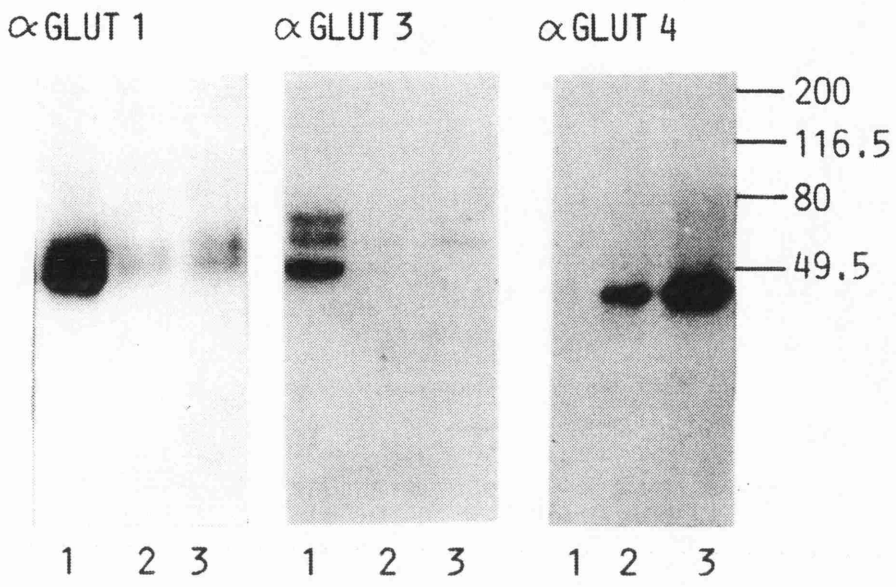


Panel a: Immunoblot analysis of mouse liver plasma membranes with anti-GLUT 2 antibodies. Plasma membranes were prepared from mouse liver as described previously (see 2.9.1). 5μg of membrane protein was electrophoresed and proteins were transferred to nitrocellulose membranes. Membranes were then probed with affinity purified anti-GLUT 2 antibodies as described before (see 2.5.1).

Panel b: Immunoblot analysis of mouse liver plasma membranes. Samples corresponding to 15 and 30μg of mouse liver plasma membranes (lanes 1 and 2) were immunoblotted with affinity purified anti- GLUT 3 antibodies and the signals obtained were compared to those obtained in 30μg and 10μg of mouse brain membranes (lanes 3 and 4 respectively).

FIGURE 3.5

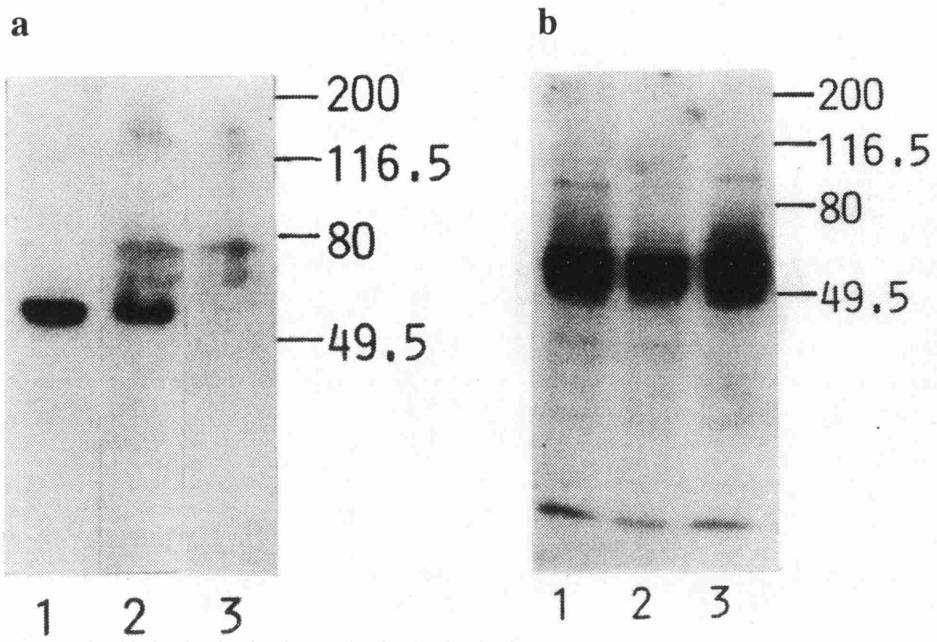
Tissue distribution of GLUT 3, GLUT 4 and GLUT 1 in mouse skeletal soleus muscle membranes



The photographs above show the results of a series of immunoblots using anti-GLUT 1, anti-GLUT 4 or anti- GLUT 3 antibodies. In each case samples contain 10μg mouse brain membranes (lane 1) 50μg mouse soleus muscle membranes (lane 2), and 100μg soleus muscle membranes (lane 3). The entire running gel is shown and the positions of the molecular weight markers in kilodaltons are shown to the right of the gel.

FIGURE 3.6

GLUT 3 expression in murine neuroblastomas



Panel a: Immunoblot analysis of murine neuroblastoma membranes with anti-GLUT 3 antibodies. Samples contain 10µg mouse brain membranes (lane 1), 25µg neuroblastoma x glioma NG 108 membranes (lane 2), 25µg rat glioma cell membranes (lane 3). The samples were probed with affinity-purified anti- GLUT 3 antibodies and the entire running gel is shown. The positions of the molecular weight markers in kilodaltons are shown to the right of the gel.

Panel b: The immunoblot shown here contains identical membrane samples to those specified in panel a but the immunoblot was developed using anti-GLUT 1 antibodies. The positions of the molecular weight markers are shown in kilodaltons to the right of the gel.

3.4 DISCUSSION

In humans, Northern blot analysis has revealed the expression of GLUT 3 in many tissues with the highest levels being detected in the brain. Many other tissues however, which contribute significantly to whole body glucose homeostasis such as fat, muscle and liver, also express GLUT 3 at the mRNA level (Kayano *et al.*, 1988).

The recent cloning of the murine homologue of this isoform has allowed anti-peptide antibodies to be produced which are highly specific for GLUT 3 protein. The production of this antibody has therefore allowed the examination of GLUT3 protein distribution in these important tissues and many others. As shown, the antibody recognised a protein at 50 kilodaltons in mouse brain membranes. The antibody also cross-reacted weakly with proteins of higher molecular weight, but these were unlikely to be glucose transporter proteins, since they could not be competed out when mouse brain membranes were immunoblotted and probed with anti-GLUT 3 antiserum containing 5μg/ml GLUT 3 peptide.

Glycosidase digestion, followed by electrophoresis and immunoblotting, indicated that the GLUT 3 exhibited increased electrophoretic mobility and therefore decreased molecular weight. This showed that GLUT 3 protein contained an N-linked oligosaccharide, a characteristic feature of facilitative-type glucose transporters.

Immunoblot analysis of 3T3-L1 murine adipocyte cell line membranes or murine liver and muscle membranes showed that no GLUT 3 immunoreactivity was present. These data were surprising since Northern blot analysis of human tissues has revealed the presence of GLUT 3 mRNA in many tissues with levels being highest in the brain, but levels being readily detectable in placenta, liver, fat, and also muscle. GLUT 3 mRNA has been shown to have a more restricted tissue distribution in the mouse compared to the human with expression being readily detectable only in brain. However using a PCR-based approach Nagamatsu *et al.* were able to show

that GLUT 3 mRNA was detectable at very low levels in lung, heart, liver and spleen (Nagamatsu *et al.*, 1992).

Taken together, these data suggest that the expression of GLUT 3 may be restricted to a smaller subset of tissues and the protein translational processes are highly regulated.

The possibility that GLUT 3 protein expression was confined to neuronally derived cells was examined. Results showed that GLUT 3 was expressed in the neuroblastoma x glioma cell line, a hybrid derived from rat glioma and murine neuroblastoma cell lines:- no GLUT 3 immunoreactivity was detected in the rat glioma parental cells whereas GLUT 1 was readily detected in both these cell lines.

These results indicate that the expression of GLUT 3 in mice is restricted to the brain and neurally derived tissues. It may be that the expression of GLUT 3 which has been observed in liver, muscle and fat from humans may be due to a richer supply of neuronal elements to these tissues in higher mammals or perhaps a reflection of neural contamination in membrane preparations.

It is well established that the major glucose transporter isoform expressed at the blood-brain barrier and the blood-nerve barrier is GLUT 1, the erythrocyte transporter (Gerhart *et al.*, 1989; Froehner *et al.*, 1988). In the brain, under normal conditions, the capacity of hexokinase for glucose is much greater than the apparent capacity of the transport system, and glycolysis is regulated by hexokinase activity and not by glucose transport into the brain cells. (Clarke *et al.*, 1981). However, it may be speculated that, under conditions of high glucose demand or hypoglycaemia, the expression of an additional facilitative glucose transporter in the brain, with a low K_m for hexoses, would ensure an efficient uptake of glucose, even when the blood glucose was low.

These results support those of Yano *et al.* (1991), who observed no expression of the GLUT 3 isoform in murine tissues other than the brain by Northern blot blotting using the human homologue GLUT 3 cDNA as a probe.

Consequently, these results suggest that the factors regulating the tissue-specific expression of GLUT 3 in humans and mice differ and that GLUT 3 expression may be controlled at defined points during transcription and/or translation.

THE IMMUNOLOGICAL ANALYSIS OF GLUCOSE TRANSPORTERS EXPRESSED IN DIFFERENT REGIONS OF THE RAT BRAIN AND CENTRAL NERVOUS SYSTEM

3.5 INTRODUCTION

In situ hybridisation analysis of GLUT 3 in the mouse brain has indicated that the expression of this isoform is not homogeneous throughout all regions of the brain (Nagamatsu *et al.*, 1992). Similar results have been reported for monkey brain, with high levels of GLUT 3 mRNA detected in the frontal lobe of the cerebrum, and lower levels recorded in the parietal lobe of the cerebrum, hippocampus and cerebellum (Yano *et al.*, 1991). In contrast, GLUT1 was expressed at highest levels in the basal ganglia and thalamus (Yano *et al.*, 1991). These studies may be taken to imply that GLUT 1 and GLUT 3 protein may also be expressed at different levels within different regions of the brain.

The sequences of mouse and rat GLUT 3 are similar and it has been demonstrated that antibodies directed against amino acids 474-493 of mouse GLUT 3 are able cross-react with rat GLUT 3 protein (Brant *et al.*, 1993). Employing this antiserum, studies carried out in rat brain showed that GLUT 3 protein was detectable in similar amounts in membranes prepared from cerebral cortex, caudate, cerebellum and hippocampus. However in the pituitary gland, GLUT 3 was detected only in the neurohypophysis, which consists of nerve fibre tracts but not in the adenohypophysis, which consists primarily of epithelial and neurosecretory cells. In contrast, however, mRNA for mouse GLUT 3 was detected in both regions of the pituitary suggesting suppression of GLUT 3 translation in the adenohypophysis (Maher *et al.*, 1992).

It has been determined that neurons are polarised cells where cell-specific sorting of proteins can occur. This sorting mechanism was demonstrated by Mantych

et al. (1992) who observed that GLUT 3 was targeted to neuronal axons alone within adult human cerebral and cerebellar cortices and furthermore, that its expression was restricted to selective adult neuronal cell bodies. These findings further support the suggestion that GLUT 3 may be expressed only in defined cell-types within specific brain regions.

Most studies in brain have focused on GLUT 1 and GLUT 3 and little is known about the regional distribution of other glucose transporter isoforms in brain.

The lateral hypothalamic area and ventromedial hypothalamic nucleus are areas of the brain which are important in the regulation of feeding (Mizuno and Oomura, 1984). It has been shown that 25% of the neurons in these regions exhibit chemosensitivity and that their activity is influenced by glucose, free fatty acids, insulin and other endogenous metabolites (Oomura, 1976). In recent years it has been reported that glucose responding neurons may also be present in other brain regions (Kadekaro *et al.*, 1980; Ritter *et al.*, 1981; Saller and Chiodo, 1980). Consequently it may be speculated that other glucose transporter isoforms such as GLUT 4, the insulin-responsive glucose transporter and indeed GLUT 2, the isoform proposed to have a role in glucose sensing in β-cells of the pancreas, may be expressed in these different regions and contribute to glucose disposal in the brain.

Membranes were prepared from individually dissected regions of rat brain and immunoblotted with antibodies specific for GLUTs 1-4 in order to examine the distribution and protein levels of these transporters within these regions.

3.6 METHODS

3.6.1 Preparation of rat brain membranes

Rat brain membranes, prepared as described below, were a gift from Dr. Christine Brown, Department of Pharmacology, Syntex Research Centre, Edinburgh.

Rat brain regions were dissected on ice immediately after sacrifice and snap frozen in liquid nitrogen. Subsequently, these regions were homogenised with 10 volumes of buffer A (10mM Tris-HCl, 0.1mM EDTA, pH 7.5) and the homogenates were centrifuged at 500 x g for 10 min. The supernatants from this process were further centrifuged at 48,000 x g for 10 min and the pellets from the second centrifugation were washed with buffer A and re-centrifuged at 48,000 x g for 10 min. Finally the pellets were resuspended in buffer A at a protein concentration of 4mg/ml and stored at -80°C. Protein concentrations were determined by the method according to Lowry *et al.* (1951).

The following rat brain regions were obtained :- frontal cortex, parietal cortex, caudate putamen, globus pallidus, hippocampus, olfactory bulb, optic chiasma, pituitary, thalamus, hypothalamus, cerebellum and medulla.

3.6.2 Immunoblotting of rat brain regions

Rat brain membranes were electrophoresed and immunoblotted as previously described (see 2.3, 2.4, 2.5.1, 2.5.2) and membranes were probed with GLUT 3, GLUT1, GLUT2 and GLUT4 antisera. Nitrocellulose membranes probed with GLUT 1 and GLUT 3 antisera were developed with ^{125}I-goat anti-rabbit IgG secondary antibody (see 2.6.1), while membranes probed with GLUT 2 and GLUT 4 antisera were developed using the ECL detection system (see 2.6.2). Quantitation of GLUT 1 and GLUT 3 immunoblot signals was carried out by carefully excising

the appropriate regions of the nitrocellulose and counting in a γ-counter. It was not possible to quantitate GLUT 4 or GLUT 2 immunoblot signals using this method since the level of these transporters was very low and required immunoblot development using the ECL detection system.

3.7 RESULTS

3.7.1 GLUT 1 protein levels and relative quantitation

The data show that GLUT 1 was expressed as a broad band at 50kDa, detectable in all regions of the brain examined (Fig 3.7). When the levels of GLUT 1 were quantitated and expressed relative to the level measured in the frontal cortex, the trend was such that the level of GLUT 1 increased towards the more posterior brain regions (see Table 3.1 and Fig 3.8). However, it should be noted that the level of GLUT 1 expression was lowest, relative to the frontal cortex, in the caudate putamen. Also the mobility on SDS-PAGE of GLUT 1 protein in the optic chiasma was decreased relative to other regions which presumably reflected differential glycosylation of this species.

3.7.2 GLUT 3 protein levels and relative quantitation

GLUT 3 was expressed as a broad band at 50kDa in all fractions examined (Fig 3.7). When GLUT 3 levels were quantitated as previously described and expressed relative to the level measured in the frontal cortex, it was observed that there was a general trend towards a decreased level of expression in posterior brain regions (see Table 3.1 and Fig 3.9).

3.7.3 GLUT 2 and GLUT 4 expression in rat brain regions

GLUT 4 was detected in three fractions :- pituitary, hypothalamus and cerebellum (Fig 3.7). This was a surprising observation since GLUT 4 is normally expressed only in tissues which exhibit acute insulin-stimulated glucose transport (see chapters 4, 5 and 6). It should be noted however, that nitrocellulose membranes probed with GLUT 4 antiserum were developed using the ECL detection system, since this is a more sensitive method which facilitates the detection of very low

amounts of protein. Thus levels of GLUT 4 detected were very low and as a result, accurate quantitation using ^{125}I-goat anti-rabbit IgG could not be performed.

GLUT 2 protein was observed in all rat brain fractions analysed here, although levels were highest in the pituitary and optic chiasma (Fig. 3.7). Again, the levels of GLUT 2 were too low to be quantitated reliably using ^{125}I-goat anti-rabbit IgG secondary antibody.

TABLE 3.1

Tables 3.1a and 3.1b below show the relative levels of GLUT 1 and GLUT 3 in rodent brain regions. Rat brain region samples were electrophoresed on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. The membranes were then probed with anti-GLUT 1 or anti-GLUT 3 antibodies and the blots were developed using ^{125}I goat anti-rabbit IgG and autoradiography. Quantitation of GLUT 1 and GLUT 3 signals was carried out by excising the approriate region of the nitrocellulose and counting in a γ-counter.

Table 3.1(a)

QUANTITATION OF THE RELATIVE LEVELS OF GLUT 1 IN RODENT BRAIN REGIONS

RAT BRAIN REGION	% EXPRESSION IN FRONTAL CORTEX
FRONTAL CORTEX	100.0
PARIETAL CORTEX	115.0 ± 3.5
CAUDATE PUTAMEN	41.5 ± 3.5
GLOBUS PALLIDUS	113.5 ± 2.1
HIPPOCAMPUS	116.0 ± 1.4
OLFACTORY BULB	135.5 ± 2.1
OPTIC CHIASMA	124.0 ± 1.4
PITUITARY	105.0 ± 3.5
THALAMUS	120.0 ± 2.1
HYPOTHALAMUS	144.5 ± 0.7
MEDULLA	109.5 ± 6.3
CEREBELLUM	171.0 ± 2.8

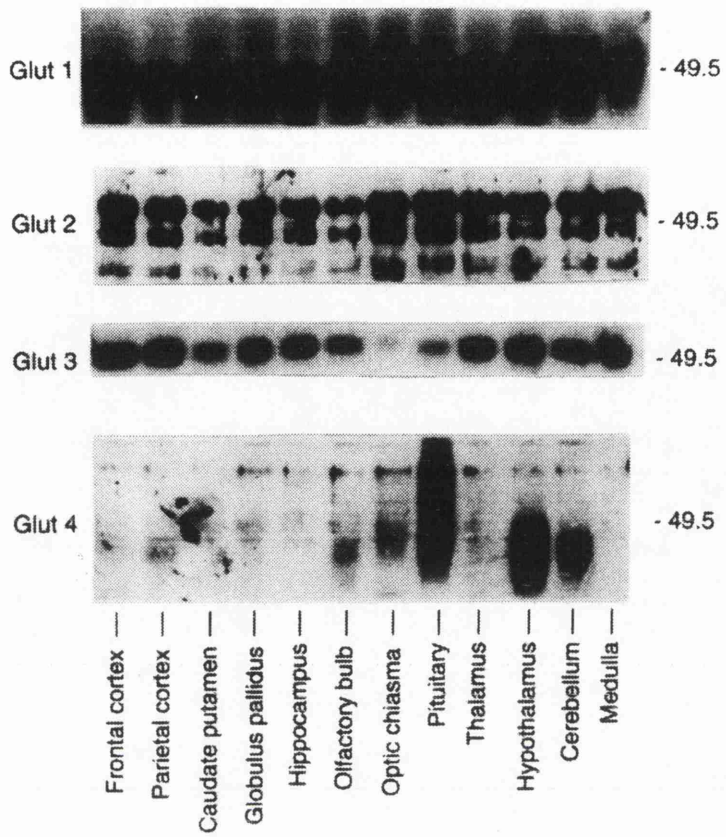
Table 3.1(b)

QUANTITATION OF THE RELATIVE LEVELS OF GLUT 3 IN RODENT BRAIN REGIONS

RAT BRAIN REGION	% EXPRESSION IN FRONTAL CORTEX
FRONTAL CORTEX	100.0
PARIETAL CORTEX	104.0 ± 7.0
CAUDATE PUTAMEN	47.0 ± 11.0
GLOBUS PALLIDUS	70.0 ± 7.7
HIPPOCAMPUS	53.5 ± 5.0
OLFACTORY BULB	49.0 ± 1.4
OPTIC CHIASMA	17.5 ± 0.7
PITUITARY	21.3 ± 1.1
THALAMUS	64.0 ± 14.1
HYPOTHALAMUS	65.5 ± 6.4
MEDULLA	65.0 ± 3.0
CEREBELLUM	42.0 ± 13.1

FIGURE 3.7

Immunological analysis of rat brain regions



Membrane proteins (25μg per lane) from the indicated rat brain regions were separated on SDS-PAGE and transferred to nitrocellulose membranes. The nitrocellulose membranes were then probed with antibodies to GLUT 1, rodent GLUT 3, rat GLUT 2 or GLUT 4 as indicated. Immunoblots probed with anti-GLUT 3 and anti-GLUT 1 antibodies were developed with ^{125}I-goat anti-rabbit IgG and the immunoblot signals were quantitated by excising the appropriate regions of the immunoblot and counting in a γ-counter (see Table 3.1). Immunoblots probed with anti-GLUT 2 and anti-GLUT 4 antibodies were developed using the ECL system to facilitate the detection of very low levels of protein. The relative position of the 49.5kDa molecular weight marker is indicated to the right of each panel.

FIGURE 3.8 **GLUT 1 distribution in brain regions.**

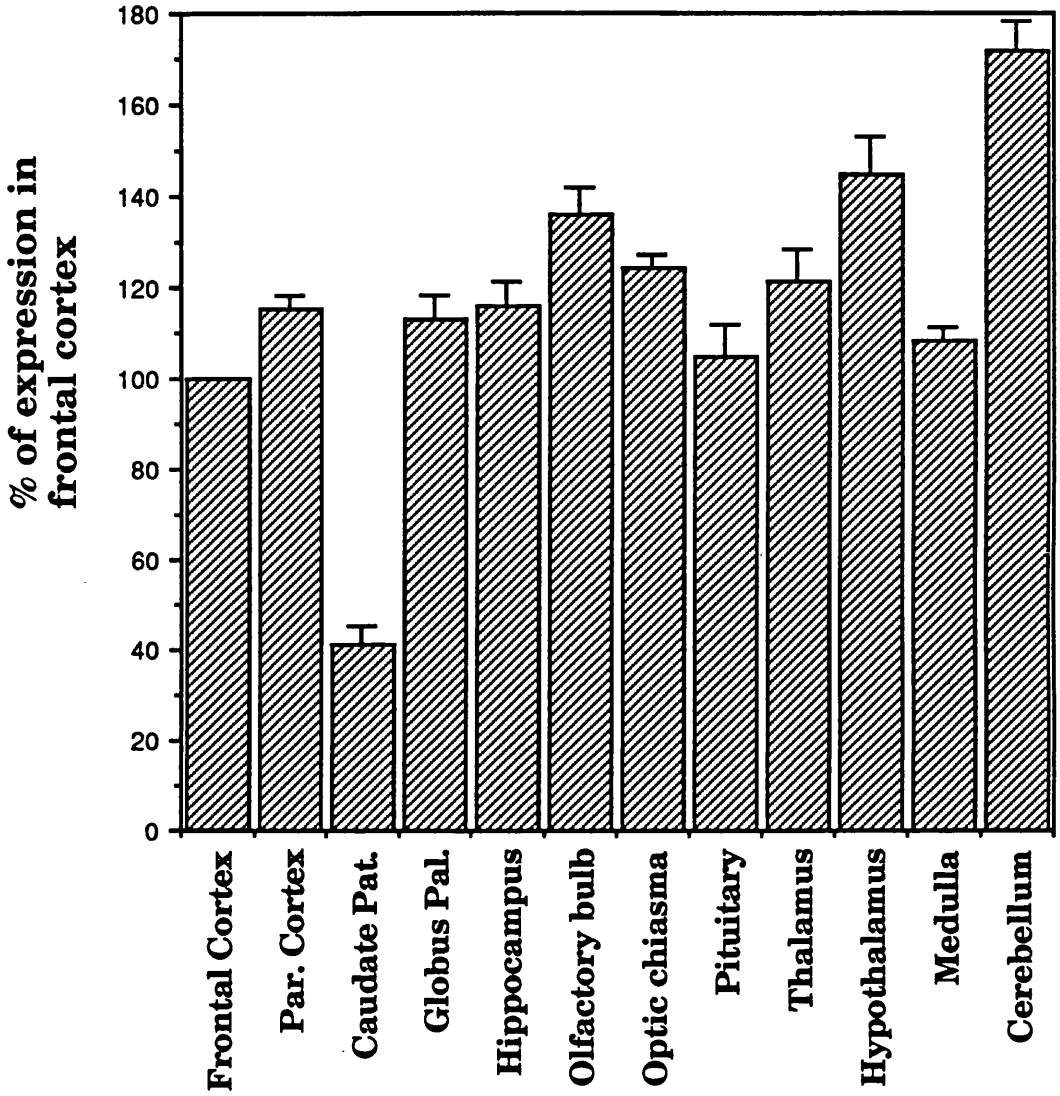
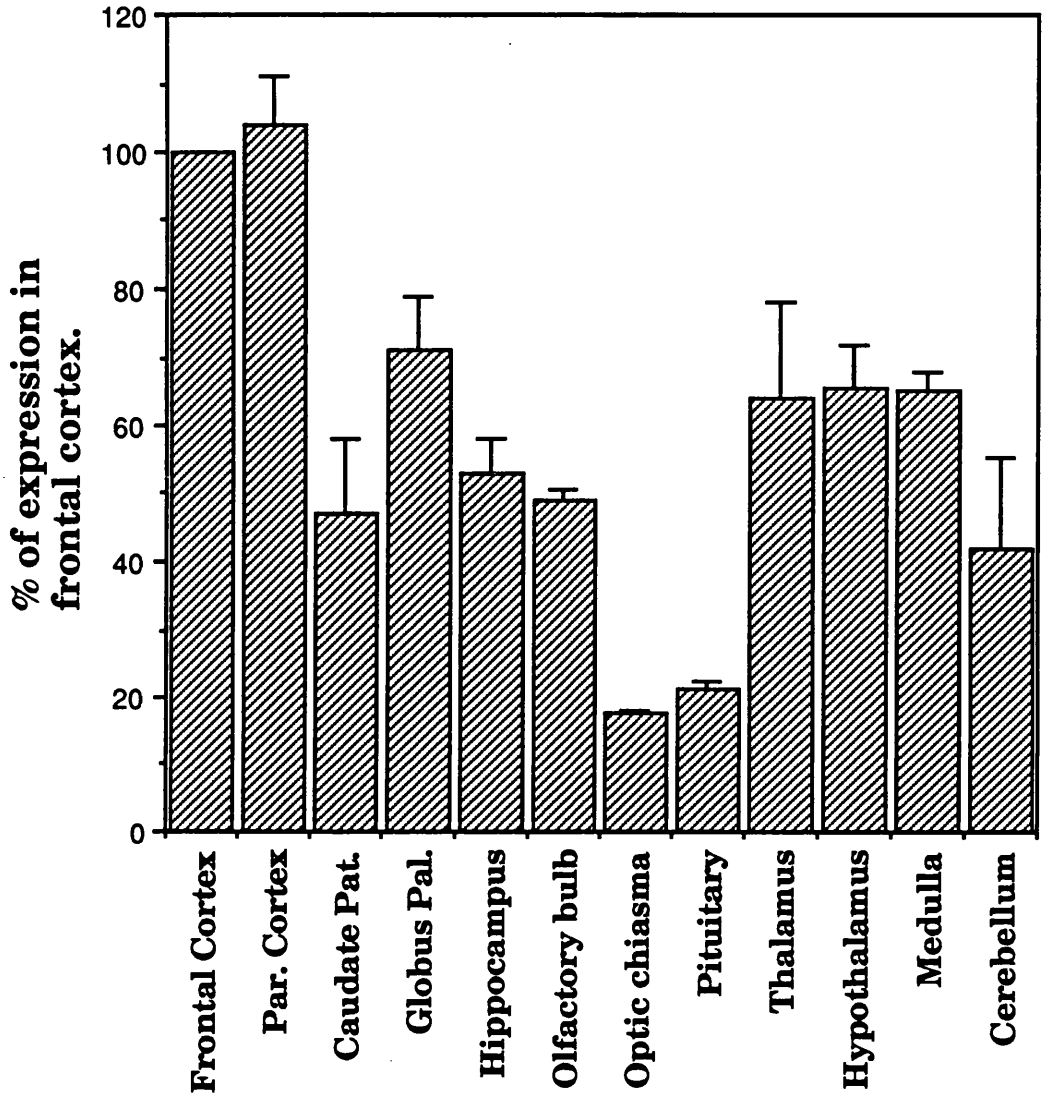


FIGURE 3.9 **GLUT 3 distribution in brain regions**



3.8 DISCUSSION

Measurement of 2 deoxy-D-glucose uptake in human brain has indicated that this process undergoes precise regulation. Furthermore, there is clearly heterogeneity in glucose utilisation among the different regions of the brain which is related to their individual metabolic demands (Phelps *et al.*, 1976; Huang *et al.*, 1980).

The results of this study imply that the different rat brain regions examined here also exhibit distinct glucose demands and/or are glucose-sensitive since they express distinct glucose transporter isoforms.

GLUT 2 was detectable in all brain regions analysed here which contradicts the observations of Widmaier who found no evidence for the expression of pancreatic β-cell-like "glucose sensors" in rat brain (Widmaier, 1992). It has been shown that regions within the hypothalamus, as yet not clearly defined, are glucose-sensitive and in addition, are intimately involved in the regulation of neurohormone release. One region, the tractus solitarius, has been proposed to be involved in the feedback regulation of neurohormone release since its activity is depressed by the local application of glucose (Mizuno and Oomura, 1984). The precise mode in which glucose exerts its inhibitory response in this region is not yet known. The brain can be likened to the pancreas in terms of its response and sensitivity to changes in glucose and metabolites of glucose. The difference however is that while glucose activates secretion in the pancreas it seems to be inhibitory for neurohormone secretion at the same concentrations in the brain (Widmaier *et al.*, 1988). Results presented here indicate that GLUT 2 was detectable in the hypothalamus in addition to other brain regions, therefore it may be that this isoform is intimately involved in glucose sensing and in co-ordinating neurohormone secretion in this region.

GLUT 4 was detectable both in the pituitary and hypothalamus. These regions of the brain are located outside the blood-brain barrier (personal communication Dr. C. Brown), therefore it is quite possible that insulin may regulate or influence glucose transport in these regions since the hormone would not have to negotiate the selectivity of the blood-brain barrier (Mizuno and Oomura, 1984). It has

been reported that various anatomical areas, including the hypothalamic regions, possess specific insulin receptors (Havrankova *et al.*, 1978). In spite of these studies however, the cellular and biochemical actions of insulin in the brain are poorly understood. Masters *et al.*, using various cell culture techniques to culture neuronal and glial cells from neonatal rat brain, showed that insulin receptors were present on neuronal cells but that they were structurally, physiologically and biochemically different from the receptors on astrocyte glial cells. Furthermore, insulin was found to stimulate 2 deoxy-D-glucose uptake in astroglial cells. They hypothesised that the receptors on astrocytic glial cells were important in more "classical" actions of insulin such as glucose uptake (Masters *et al.*, 1987).

Results here indicate that GLUT 4 was detectable in the hypothalamus which suggests that this isoform may indeed be involved in glucose uptake in response to insulin. It could be hypothesised that GLUT 4 responds to insulin by translocating from one region of the cell to the plasma membrane upon stimulation, thus promoting increased glucose uptake.

GLUT 3 protein levels were lowest towards the posterior regions of the brain where GLUT 1 levels were highest. Therefore it would appear that a low level of one transporter isoform is compensated for by a higher level of the other. This may represent an adaptation to ensure efficient glucose uptake into all brain regions.

The brain is an organ involved in many diverse processes and the role of each glucose transporter in overall brain metabolism cannot be delineated until more information regarding glucose transporter expression is obtained. The regulation of glucose transporter expression during brain development and targeting in specific cell-types within gross brain regions must be studied in more depth to facilitate a greater understanding of brain glucose transport. Nevertheless, the data presented here have provided some evidence, albeit speculative, that glucose transporter isoform expression in the brain regions is region-specific and that their expression may be tightly controlled.

CHAPTER 4

THE EXPRESSION OF GLUT 2 IN 3T3-L1 FIBROBLASTS

4.1 INTRODUCTION

GLUT 2 and GLUT 4 are both facilitative-type glucose transporters which are expressed in insulin-responsive tissues. As previously mentioned in preceding sections, GLUT 2 is expressed in the kidney, the β-cells of the pancreas, where it is proposed to have a role in glucose-sensing and in the liver where it mediates the bi-directional movement of glucose both into and out of hepatocytes. GLUT 4, in contrast, is expressed in insulin-sensitive tissues, fat, muscle and heart and, upon exposure to insulin moves from an unidentified intracellular location to the plasma membrane by a process termed translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Slot *et al.*,1991a). Many studies have been carried out in an attempt to identify the specific region to which GLUT 4 protein is sequestered. A very recent study by Martin *et al* (in press) however, has shown that the *trans* -Golgi network, a compartment known to be involved in membrane protein recycling is not the major intracellular sequestration site of GLUT 4 within 3T3-L1 adipocytes. Using immunoadsorption and immunofluorescence techniques, Martin and co-workers showed that TGN38, a specific marker for the *trans* -golgi network, did not co-localise with GLUT 4 thus demonstrating that the transporter was localised to a different compartment. Consequently the intracellular pool to which GLUT 4 is localised remains to be defined (Martin *et al.*, in press).

Studies in both isolated rat adipocytes and the murine 3T3-L1 clonal cell line have demonstrated that in resting adipocytes, approximately 90% of the GLUT 4 molecules are located intracellularly and upon exposure to insulin about 40% of these transporters move to the cell surface and account for most, if not all, of the increased

glucose transport observed in response to insulin (Zorzano *et al.*, 1989; Holman *et al.*, 1990; Calderhead *et al.*, 1990; Piper *et al.*, 1992 ; Slot *et al.*, 1991a).

Although GLUT 2 and GLUT 4 are both expressed in insulin-responsive tissues, liver and fat respectively, insulin stimulation of resting hepatocytes does not stimulate glucose transport and by inference, GLUT 2 does not undergo insulin-induced translocation (Thorens *et al.*, 1988; Fukomoto *et al.*, 1988).

It has been shown by expressing cDNAs encoding GLUT 2 and GLUT 4 in *Xenopus* oocytes, that each transporter possesses a different affinity for cytochalasin B, an inhibitor of sugar transport and, in addition, each exhibits a different transport K_m for glucose. The low K_m of GLUT 4 for glucose ensures that it functions close to its V_{max} under normal physiological conditions where blood glucose values are approximately 5mM. GLUT 2 has a higher transport capacity compared with GLUT 4 and a much higher K_m for glucose (approximately 15mM) (Colville *et al.*, 1993). The supraphysiological K_m value of GLUT 2 thus ensures that the transport rate is never rate-limiting for glucose metabolism. This system allows free equilibration of glucose inside the cell and glucose in the extracellular blood and consequently, fluctuations in blood glucose concentrations are matched with the changes in the intracellular glucose concentration. Furthermore, it has been demonstrated by Colville *et al.* (1993) that GLUT 2 is capable of mediating the transport of D-fructose and exhibits a K_m of 67mM for this sugar.

GLUT 2 expression has been localised to the basolateral surface of the absorptive epithelial cells of the small intestine (Thorens *et al.*,1990) where it is likely to mediate the efflux of glucose and fructose from these cells. GLUT 2 is probably also responsible for the uptake of fructose by the liver since this organ is the primary site of fructose metabolism and no other specific fructose transporter has yet been identified in this tissue.

The majority of studies on glucose transporter translocation and targeting have been concerned with trying to identify which specific amino acid residues or indeed specific domains of the protein dictate targeting and/or translocation (for

review see James and Piper, 1993). A common approach to study targeting and translocation has been to express the insulin-responsive GLUT 4 protein in cells which do not exhibit increased glucose uptake upon exposure to insulin (Hudson *et al.*, 1992; Shibasaki *et al.*, 1992). Results from such studies have indicated that GLUT 4 is sequestered to an intracellular region in these cells and is not transported to the cell surface like other isoforms. Furthermore, the expressed GLUT 4 cannot be recruited to the cell surface following treatment of these cells with insulin. Several recent studies have reported sequences in the GLUT 4 molecule which are believed to be intimately involved in preventing GLUT 4 transit to the cell surface (Asano *et al.*, 1992; Piper *et al.*, 1993).

Asano *et al.* (1992) reported that the intracellular targeting domain was localised to around transmembrane regions 7 and 8, which was not in agreement with results reported by Piper *et al.* (1992) who claimed that targeting information was contained within the first 8 N-terminal amino acids. Hence it could be postulated, from these conflicting results, that possibly the targeting domain comprises more than one region and that perhaps domain-domain interactions have an important role to play in the targeting process.

Most studies on transporter targeting and translocation to date have been concerned with over-expressing GLUT 1 and GLUT 4 separately in an insulin-insensitive cell environment where neither protein is the "native" glucose transporter. A different strategy however was undertaken in the investigation described here.

This study was carried out in an attempt to determine whether translocation is a process governed by the type of cell in which the glucose transporter is expressed or whether it is a property encoded in the protein sequence itself. This work was concerned with the targeting and insulin-responsiveness of GLUT 2, the liver transporter, when expressed in a fat cell environment. By expressing GLUT 2 in a fat cell model it should be possible to determine whether the transporter retains its own distinct properties or whether it exhibits identical properties to those of the native fat cell glucose transporter GLUT 4. In other words, when expressed in a fat cell

environment does GLUT 2 undergo translocation? The 3T3-L1 cell line was used as an adipocyte cell model and studies were carried out in undifferentiated and differentiated GLUT 2-transfected cells. The first part of this study, characterising GLUT 2 insulin-responsiveness in the undifferentiated fibroblast cell is described here (see chapter 5 for GLUT 2 insulin-responsiveness in 3T3-L1 adipocytes).

4.1.1 3T3-L1 :- a murine fat cell model for the expression of GLUT 2

The 3T3-L1 cell, a murine fibroblast cell line isolated and cloned by Green and Kehinde, differentiates to an adipocyte-like cell which synthesises and stores triacylglycerols (Green and Kehinde 1974; Green and Kehinde 1975; Green and Kehinde, 1976). Fibroblast cells spontaneously generate foci of adipocytes when maintained at confluence in culture for several weeks. However, differentiation can be artificially induced by exposing 3T3-L1 fibroblasts, 2 days post confluence, to 0.25μM dexamethasone, 0.5mM methyl isobutylxanthine and 1μg/ml insulin (see 2.8.4 and 2.8.5). During fibroblast differentiation to adipocytes, the number of insulin receptors per cell increases approximately 35 -fold (Rubin *et al.*,1978) and as a result glucose transport becomes highly sensitive to insulin (Rosen *et al.*, 1978; Resh, 1982). Therefore the 3T3-L1 cell line has been widely used as a model to study the molecular basis of insulin action on glucose transport.

3T3-L1 cells express two facilitative-type glucose transporter isoforms (James *et al.*, 1989; Kaestner *et al.*, 1989). The erythrocyte/brain glucose transporter, GLUT 1 is present both in undifferentiated and differentiated cells, whereas the muscle/adipose glucose transporter, GLUT 4, is expressed only when the cells are in the differentiated state. It has been reported that the large response to insulin in glucose transport activity correlates with the appearance of Glut 4 protein in 3T3-L1 adipocytes (deHerreros and Birnbaum, 1989). Shibasaki *et al.* (1992) showed that glucose uptake in fibroblasts increased 2 -fold in response to insulin whereas transport activity in adipocytes increased greater than 10 -fold upon exposure to

insulin. Consequently it was suggested that the lack of response to insulin of fibroblasts resulted from the absence of GLUT 4 protein.

Transfection studies carried out by Shibasaki *et al.* (1992) in GLUT 4-transfected 3T3-L1 fibroblasts, indicated that the expressed protein did not appear to contribute to glucose transport activity in the presence or absence of insulin. Therefore from these findings it seemed that the expression of the insulin-responsive GLUT4 transporter alone was not adequate to confer insulin-sensitivity and that factors which are present in the adipocyte form of the cell may be necessary for a large increase in glucose transport activity to be observed upon insulin stimulation. Following differentiation of these GLUT4-transfected 3T3-L1 cells to adipocytes and insulin exposure, a large increase in glucose uptake was observed, however investigators were unable to demonstrate that transfected GLUT 4 protein itself responded to insulin since the transfected GLUT 4 could not be distinguished from endogenous GLUT 4 protein also present in the cells (Shibasaki *et al.*, 1992).

Hudson *et al.* (1992) carried out similar studies using NIH-3T3 cells and confirmed the findings of Shibasaki *et al.* (1992) as outlined above. Investigators demonstrated, by overexpressing GLUT 1 and GLUT 4 in these cells, that the expression of GLUT 4 alone was not adequate to confer insulin-responsive glucose transport. Results indicated that, in GLUT 1-transfected cells, basal glucose uptake was increased relative to control cells and that the expressed protein was plasma membrane- localised. Furthermore, insulin-stimulated 2-deoxyglucose uptake was increased in GLUT 1-expressing cells but the fold increase over basal was identical to that observed in control cells. Also it was demonstrated that neither basal nor insulin-stimulated rates of glucose uptake were increased in GLUT 4-transfected cells relative to control cells. Immunofluorescence data showed that GLUT 4 was predominantly intracellularly localised to an endocytic compartment, since GLUT 4 immunofluoresence staining co-localised with markers for early and late endosomes, the transferrin receptor and mannose-6-phosphate receptor, respectively.

On the basis of these results it could be hypothesised that GLUT 2 when expressed in fibroblasts would be correctly targeted to the plasma membrane. In addition, it would seem unlikely that the transfected protein would be insulin-responsive since previous studies have shown that even GLUT 4, the adipocyte "insulin-responsive" glucose transporter does not exhibit translocation when expressed in a fibroblast cell environment.

This study was carried out to investigate the validity of these speculations and to determine if GLUT 2 could contribute to sugar uptake when expressed in 3T3-L1 fibroblasts.

4.1.2 Expression of GLUT 2 in 3T3-L1 fibroblasts

As a first step in an attempt to study the properties of GLUT 2 in a fat cell environment, 3T3-L1 fibroblasts were transfected with an expression vector containing the cDNA encoding the full length human GLUT 2 protein.

Under the appropriate conditions, eukaryotic cells can take up exogenous DNA, and a portion of this DNA can become localised in the nucleus and incorporated into the host cell chromosome. However, the spontaneous entry of intact DNA into the cell and its subsequent expression in the nucleus is a very inefficient process.

A large variety of methods including calcium phosphate (Graham and Van der Eb, 1973; Loyter *et al.*, 1982), liposome fusion (Cudd and Nicolau, 1984), retroviral-mediated transfer (Cepko *et al.*, 1984), microinjection (Graessmann and Graessmann, 1986), electroporation (Neuman *et al.*, 1982) and protoplast fusion (Schaffer, 1980) have been developed to facilitate this process. However, all of these methods suffer from one or more problems related to either cellular toxicity, poor reproducibility, inconvenience or inefficiency of DNA delivery.

Recently, a delivery reagent was developed by Felgner *et al.* (1987) which consists of polycationic liposomes comprising a positively charged lipid named

DOTMA (N [1-2, 3-dioleyloxy) propyl]-N,N,N-triethyl-ammonium). This reagent has circumvented most of the problems mentioned previously and is convenient to use. DNA/DOTMA complexes form spontaneously when an aliquot of the reagent is mixed with an aqueous solution of DNA, and the complex, having a net positive charge, is then able to interact with the negatively charged cell plasma membrane thus facilitating DNA entry into the cell.

Since the development of DOTMA, modified forms of the transfection reagent, such as DOTAP, (N-[1-(2,3-Dideolyloxy)propyl]-N,N,N-triethyl-ammonium-methyl-sulphate) have emerged. In this study 3T3-L1 fibroblasts were transfected by lipofection using the DOTAP reagent (see 2.8.10).

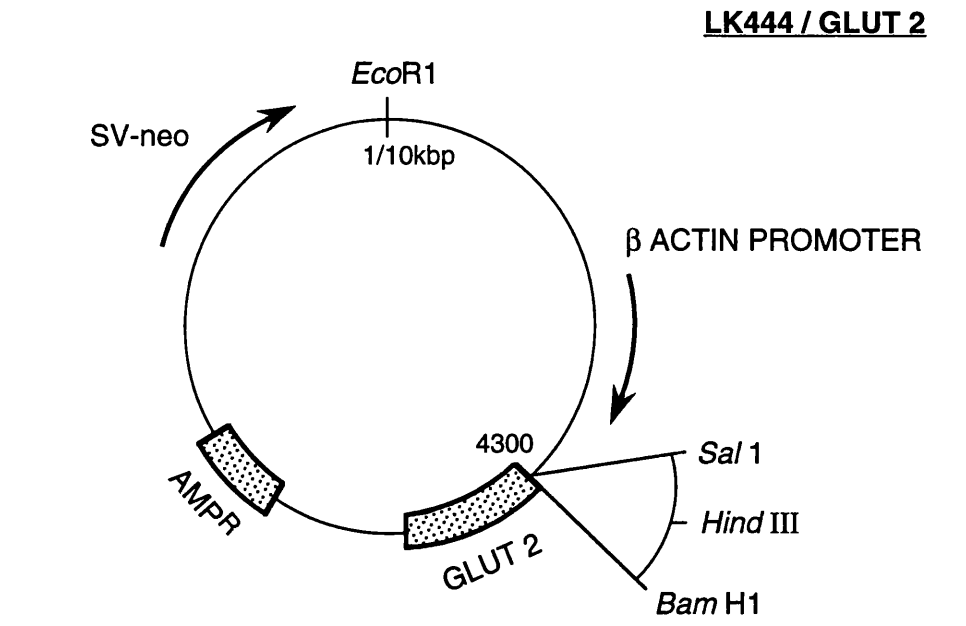
4.1.3 The expression vector

The vector selected for the expression of GLUT 2 in fibroblasts was LK444, which encompasses the human β-actin promoter linked to 3 unique restriction sites and a simian virus (SV40) polyadenylation signal (see Fig. 4.1). This vector, developed by Gunning *et al.*,(1987) is a high efficiency expression vector which utilises the β-actin promoter to drive the expression of cloned cDNA. Since β-actin is one of the most abundant of mRNA species in all non-muscle cells it would be expected that this promoter would be highly active all non-muscle cells. Also contained in the plasmid is the neomycin resistance gene which allows for the selection of transfected cells in culture. Cells which have taken up the plasmid can grow and survive in culture medium containing the antibiotic, whereas non-transfected cells cannot.

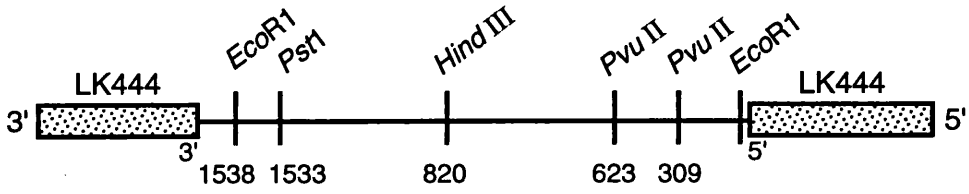
3T3-L1 fibroblasts were stably transfected with the prepared LK444/GLUT 2 construct and antibiotic resistant clones were selected and expanded in culture.

GLUT 2 expressing clones were identified by the immunoblotting of lysates prepared from clones with anti-GLUT 2 antibodies. 2-Deoxy-D-glucose and D-

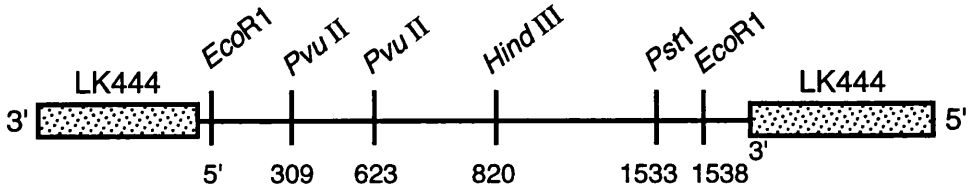
Figure 4.1



CORRECT GLUT 2 ORIENTATION



INCORRECT GLUT 2 ORIENTATION



fructose uptake was then determined in GLUT 2 expressing cells and compared with uptake measured in non-transfected fibroblasts.

Transport rates were also measured in insulin-stimulated GLUT 2-expressing cells and compared with rates measured in control cells in an attempt to determine if the expressed GLUT 2 protein exhibited insulin-sensitivity.

4.2 METHODS

4.2.1 Construction of the expression vector containing GLUT 2

pHTL.217, containing cDNA encoding the full length human GLUT 2 protein was a gift from Dr. Graeme Bell, University of Chicago.

GLUT 2 cDNA was isolated from pHTL.217 by restriction digestion. The vector was digested for 60 min at 37°C with *Bgl* II in a reaction volume of 20µl. After this time, 5µl of the isolated DNA was mixed with 5µl of 5X DNA loading buffer and the sample was electrophoresed on a 1% agarose gel to verify that the yield of DNA was sufficient to perform subsequent subcloning steps.

4.2.2 Preparation of the expression vector LK444 for ligation to GLUT 2 cDNA

LK444 plasmid contains 3 unique restriction sites, *Bam* HI, *Hind* III and *Sal* I, into which DNA fragments may be subcloned (see Fig. 4.1). In this case, the vector was digested with *Bam* HI since the "sticky" DNA ends resulting from this digestion are compatible with the *Bgl* II- digested "sticky" ends of GLUT2 cDNA generated after its isolation from the parent pHTL.217 plasmid. It should be noted, however, that when *Bgl* II-digested and *Bam* HI-digested ends are ligated together neither the *Bgl* II or the *Bam* HI sites can be regenerated since the recognition site for each enzyme is destroyed.

5µg LK444 was digested with 1 unit of *Bam* HI at 37°C for 60 min as previously described, and treated with 1µg of RNase for a further 30 min at 37°C. Following phenol extraction and ethanol precipitation (see 2.7.9), LK444 was treated with calf intestinal phosphatase (CIP) to de-phosphorylate the 5' end of the linearised DNA (see 2.7.8). This process ensured that linearised LK444 DNA would not

simply self-ligate. 1µl of CIP-treated DNA was then electrophoresed on a 1% agarose gel to verify that the DNA had been linearised.

4.2.3 Ligation of phosphatase-treated LK444 to GLUT 2 cDNA

GLUT 2 cDNA was ligated with linearised LK444 DNA at a molar ratio of 10:1 using the method described previously (see 2.7.9). Following this procedure, 200µl of competent *E. coli* cells were transformed with 1µl of the ligation mix and the cells were spread at several dilutions on 2xYT agar plates containing ampicillin at 25µg/ml (see 2.7.3). The plates were then incubated at 37°C overnight.

In addition to the ligation mix transformation reaction, control transformations were also assembled to demonstrate that *E. coli* cells were competent and to verify that the 2xYT agar plates contained ampicillin concentrations which were sufficiently potent for non-transformed *E. coli* cells. Competent *E. coli* cells were transformed with either 100ng linearised LK444 DNA alone, 100ng undigested LK444 DNA or with sterile H_2O. It would be expected that no colonies would be present on 2xYT agar/ampicillin plates spread with *E.coli* transformed with 100ng linearised LK444 since cells which had taken up this non-competent DNA would not be ampicillin resistant. Also no colony growth would be expected on 2xYT agar/ampicillin plates spread with competent cells incubated with sterile H_2O alone since no DNA was present which would confer ampicillin resistance. However, discrete bacterial colonies would be expected on 2xYT agar/ampicillin plates spread with *E. coli* transformed with 100ng undigested LK444 DNA since this plasmid would confer ampicillin resistance to transformed bacteria.

A number of ampicillin-resistant colonies were individually selected from the ligation mix 2xYT/ampicillin plate and each was used to inoculate 2ml of 2xYT medium containing 25µg/ml amipicillin. These mini-preps were grown overnight at 37°C and the following morning plasmid DNA was isolated from the *E.coli* by

alkaline lysis. The plasmid DNA was then purified by phenol extraction and ethanol precipitation (see 2.7.1).

To check the efficiency of DNA preparation, 1µl of the isolated DNA was mixed with 5µl of 5X DNA loading buffer and the sample was electrophoresed on a 1% agarose gel. A sample of *Bam* HI-linearised LK444 DNA and a sample of *Bst* E II-digested λ ladder were also electrophoresed on the gel so that the size of the isolated DNA could be estimated relative to the λ ladder fragments and to undigested circular LK444 DNA.

4.2.4 Restriction digestion of ligated DNA to determine GLUT 2 orientation.

Restriction digestion reactions were performed on ligated LK444/GLUT 2 cDNA to determine if GLUT 2 insert was present, and if so to determine its orientation within the expression vector. GLUT 2 cDNA can ligate with LK444 DNA either in a 5' to 3' or 3' to 5' orientation since the GLUT 2 "sticky" ends generated from the pHTL.217/*Bgl* II digest are identical at both 5' and 3' ends. (see Fig. 4.1 for positions of restriction sites when GLUT 2 ligated in the correct and incorrect orientation).

Restriction digestion reactions were carried out using a chosen combination of restriction enzymes and the sizes of the DNA fragments generated from each digestion reaction were calculated. Table 4.1 shows the sizes of fragments expected from each digestion reaction when GLUT 2 is ligated to LK444 in the correct (sense) or incorrect (antisense) orientation.

4.2.5 Large scale preparation of LK444/GLUT2 DNA

Once LK444/GLUT 2 DNA, with GLUT 2 in the correct orientation, had been identified, the mini-prep from which it was first isolated was used to inoculate

TABLE 4.1

To determine the orientation of GLUT 2 cDNA within LK444/GLUT 2, restriction digestion reactions were assembled as outlined below. The sizes of DNA fragments expected from LK444/GLUT 2 digestions, when GLUT 2 is ligated in the correct or incorrect orientation, are shown.

Restriction enzyme	**fragment size (bp) (correct orientation)**	**fragment size (bp) (incorrect orientation)**
Eco RI	4300, 1540, 5910	4510, 1540, 5700
Eco RI/*Hind* III	4300, 820, 720, 5700, very small fragment	4300, 210, 720, 820, 5700
Hind III	820*, 10930	930~, 197, 314, 10309
Hind III/*Pvu* II	309*, 314*, 197*, 10930	930~, 197, 314, 10309
Hind III/ *Pst* I	820, 713, 10210	217~, 713~, 10820

*Definitive digest fragment: If GLUT 2 is in the correct orientation then this 820bp fragment should be digested further to yield fragments of 309, 314, 197 in the presence of *Pvu* II.

~If GLUT 2 is in the incorrect orientation then the 930~bp fragment should remain with *Hind* III/*Pvu* II digestion but be further digested to yield fragments of 217 and 713bp in the presence of *Hind* III/*Pst* I.

500ml 2xYT medium for a large scale preparation in order to amplify the DNA. Following this, the concentration and purity of the isolated DNA was determined and restriction digestion reactions were assembled, as before, to verify that the correct construct had been isolated. The DNA was then sequenced using the Applied Biosystems sequencing system.

4.2.6 Sequencing of LK444/GLUT 2 by automated sequencing

The *Taq* DyeDeoxy^{TM} Terminator Cycle Sequencing Kit was used for sequence analysis of LK444/GLUT 2 DNA on the Applied Biosystems sequencing system (model 373A).

The DNA was prepared as described in the manufacturers manual but in brief terms, 0.5µg DNA was mixed with 0.8 pmols of the appropriate oligomer and the volume was made up to 10.5µl with distilled H_2O. This was then transferred to a 0.5µl Eppendorf tube and 9.5µl of reaction mix, containing 4µl of 5X Tacs buffer (400mM Tris-HCl, 10mM $MgCl_2$, 100mM $(NH_4)_2$ SO_4, pH 9.0), 1µl of dNTP mix, 1µl each of Dye Deoxy^{TM} A terminator, G terminator, C terminator, and T terminator and 0.5µl of Amplitaq DNA polymerase, was added. DNA was then amplified in a PCR machine on the recommended program for 25 cycles and following this, was cleaned by phenol/chloroform extraction and ethanol precipitation (see 2.7.1).

This procedure was in accordance with the ABI protocols for use with the Applied Biosystems Automated Sequencer.

4.2.7 Determination of G418 concentration for the selection of transfected 3T3-L1 fibroblasts

LK444, as previously mentioned, contains the neomycin resistance gene which can be utilised for the selection of transfected mammalian cells in culture since

cells which have taken up the plasmid can grow and divide in culture medium containing Geneticin (G418-sulphate, a derivative of neomycin), whereas cells which have not taken up the plasmid cannot. To determine a suitable concentration of G418 in which to maintain fibroblasts after transfection, wild-type 3T3-L1 cells at confluence were maintained in 10% calf serum/DMEM medium containing 0, 0.25, 0.5, 0.8, 1.0, or 1.2mg/ml Geneticin. The appearance and number of surviving cells was noted each day.

Within 7 days all cells grown in 0.8-1.2mg/ml G418 had perished while those in 0-0.5mg/ml appeared healthy. However, after 9-12 days cells grown in 0.25 and 0.5mg/ml G418 began to die off while cells grown in the absence of G418 continued to grow. From these data it was decided that transfected cells would be selected initially in 0.5mg/ml G418 since this concentration was lethal to non-transfected cells but its effect was relatively slow. The slow potency of the antibiotic at this concentration would allow potentially transfected cells to become established before effects of the antibiotic would be maximal. If greater concentrations of the antibiotic were used initially then the effect may be almost instantaneous and transfected cells would perish immediately. Following the appearance of discrete antibiotic resistant colonies it was decided that the G418 concentration would be reduced and that cells would be maintained in 0.25mg/ml.

4.2.8 Transfection of 3T3-L1 fibroblasts with LK444/GLUT2 DNA

3T3-L1 murine fibroblasts were grown to 70-80% confluency in 10% calf serum/DMEM medium on 10cm cell culture plates and cells were then transfected with 5μg of LK444/GLUT 2 DNA using the DOTAP transfection procedure (see 2.8.10). Two days post-transfection, 5ml of cell culture medium was carefully removed and replaced with medium containing G418 such that the final concentration of the antibiotic per plate was 0.5mg/ml. Medium containing G418 was replaced using this procedure every two days.

After 9-12 days most of the cells had perished although a number of single surviving cells were scattered over the plate. After 2-3 days, these cells began to grow and divide forming small colonies which were antibiotic resistant. Following another 5-7 days of careful feeding each colony was isolated and re-plated on a 6cm plate containing 20% calf serum/DMEM medium with G418 at a concentration of 0.25mg/ml (see 2.8.11).

The calf serum concentration in DMEM was increased from 10% to 20% in DMEM at this stage in order to maximise growth conditions for the transfected cells. Colonies were grown until 70% confluent at which time cells were trypsinised and used to seed three to five 10cm plates. These cells were then grown to confluency in 20% calf serum/DMEM medium, trypsinised, frozen down and stored at -80^{o}C as cell stocks (see 2.8.6). In addition a cell lysate was prepared from one 10cm plate for each clone so that the sample could be screened for the expression of GLUT 2.

4.2.9 Screening 3T3-L1 fibroblasts for the expression of GLUT 2 protein

Cell lysates, prepared from a number of individual G418-resistant clones, were electrophoresed on 10% SDS/polyacrylamide gels and the proteins were transferred to nitrocellulose membranes (see 2.4). The membranes were blocked in 5% non-fat milk/1st wash buffer and incubated with anti-GLUT 2 antiserum in 1% non-fat milk/1st wash buffer (see 2.3, 2.4, 2.6.1). Nitrocellulose membranes were developed using the ECL detection system so that the presence of very low levels of protein could be detected (see 2.6.2).

4.2.10 Measurement of [^{3}H]-2-deoxyglucose and [^{14}C] D-fructose uptake in transfected and non-transfected cells

Non-transfected and transfected 3T3-L1 fibroblasts were grown in 10% or 20% calf serum/DMEM respectively on 6-well plates until they had reached confluency. 2h prior to measuring sugar uptake, the cells were incubated in serum-free DMEM medium. The uptake of 0.1mM [^{3}H] 2-deoxyglucose or 1mM [^{14}C] D-fructose (0.5μCi/ml) was then measured in the absence or presence, for 30 min, of 1μM insulin (see 2.10).

4.3 RESULTS

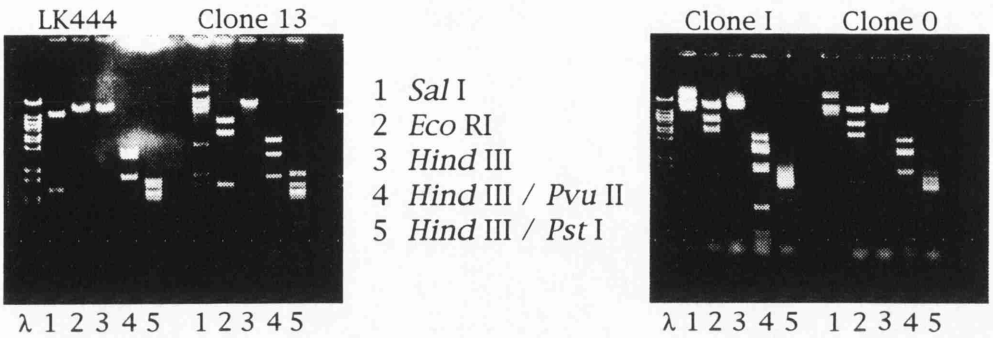
4.3.1 Restriction digestion of LK444/GLUT 2 DNA

Restriction digestion of LK444 DNA indicated that two *Sal* I restriction sites were present in the plasmid since digestion with this enzyme yielded 2 fragments of approximately 10Kbp and 1.75Kbp in size (Fig. 4.2). Restriction digestion with *Eco* RI or *Hind* III indicated that one site for each enzyme was present since each digest generated a single fragment representing the linearised form of LK444 (Fig. 4.2). *Pvu* II or *Pst* I restriction digestions yielded greater than 6 fragments which demonstrated that at least 5 sites for each enzyme were present in LK444 (Fig. 4.2).

Restriction digestion reactions, using the combination of enzymes indicated in Table 4.1, were carried out on four LK444/GLUT 2 DNA samples designated I, O, P and 13 isolated from 4 selected transformed *E.coli* colonies (see 2.7.1). Results indicated that O, I, and 13 all contained GLUT 2 DNA in the correct orientation (Fig 4.2), but that sample P contained GLUT 2 in the reverse orientation (data not shown). The evidence for this was obtained by comparing the sizes of the fragments generated from digestion with *Hind* III with those generated from digestion with both *Hind* III and *Pvu* II enzymes. A fragment of approximately 820bp in size was obtained in samples I, O, and 13 upon digestion with *Hind* III (Fig 4.2) which represented the fragment from restriction co-ordinate 4300 (in the plasmid) to co-ordinate 820 (in the GLUT 2 insert) (Fig.4.1). It would be expected that, if GLUT 2 was contained in LK444 /GLUT 2 in the correct orientation, this fragment would be further digested in the presence of *Pvu* II since 2 sites for *Pvu* II at co-ordinates 309 and 623 (in the insert) are present between the *Hind* III sites at co-ordinates 820 (in the insert) and 4300 (in the plasmid) (Fig 4.1). If however the GLUT 2 insert was in the incorrect orientation, then the 820bp fragment generated from *Hind* III digestion would remain unchanged in size in the presence of *Pvu* II, since *Pvu* II sites would now be located at co-ordinates beyond the insert *Hind* III site (Fig. 4.1).

FIG. 4.2

Restriction digestion of LK444 and LK444/GLUT 2 DNA



LK444 DNA and LK444/GLUT 2 DNA, isolated from samples 13, I and O, was subjected to restriction digestion as described in section 2.7.6. Restriction digestion reactions were assembled using the combination of enzymes specified in Table 4.1 which also shows the sizes of DNA fragments expected from each digestion reaction. The digested DNA samples were then electrophoresed on 1% agarose gels contained in gel tanks which were filled with 1X TAE buffer containing 25µg/ml ethidium bromide. Electrophoresis was carried out, the gels were viewed under U.V. light and a photograph of each was taken.

The figures above show photographs of the 1% agarose gels through which the digested DNA samples were electrophoresed. The restriction enzyme combinations used in each digest are specified to the right of each figure. The sizes in bp of the *Bst E* II-digested λ DNA fragments are as follows:- 8452, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, 224, 117.

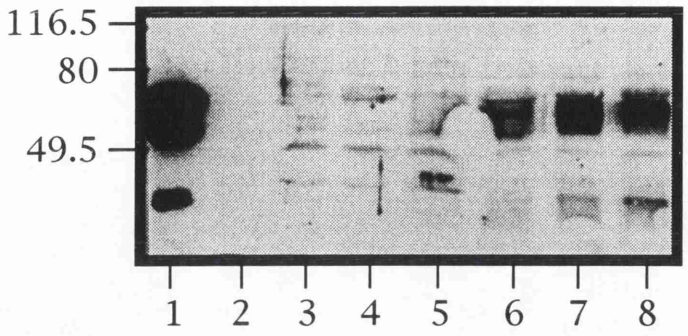
Results indicated that the 820bp fragment generated upon *Hind* III digestion in samples I, O and 13 but not P, was further digested in the presence of both *Hind* III and *Pvu* II but remained unchanged in size in the presence of *Hind* III and *Pst* 1 (Fig. 4.2). This clearly indicated that GLUT 2 cDNA was ligated to LK444 in the correct orientation in clones I, O and 13. A fragment of 930bp was generated upon *Hind* III digestion of clone P. This fragment was unchanged in size in the presence of *Hind* III and *Pvu* II but was further digested in the presence of *Hind* III and *Pst* 1 (data not shown). These results confirmed that GLUT 2 cDNA was ligated in the incorrect orientation in this clone. Data obtained from automated sequencing of LK444/GLUT 2 in samples 13, O and I confirmed that the correct GLUT 2 cDNA sequence was contained within the LK444 plasmid and that it was ligated in the correct orientation (data not shown).

4.3.2 Immunoblotting of lysates prepared from GLUT 2-transfected fibroblasts

Immunoblotting of cell lysates prepared from a number of GLUT 2-transfected antibiotic resistant clones with affinity purified anti-GLUT 2 antibodies identified a major band at 55 kilodaltons in 2 clones designated 3T3/G2a and 3T3/G2b (Fig 4.3). No GLUT 2 expression was detectable in clone 3T3/ca or in wild type non-transfected 3T3-L1 fibroblasts. Immunoblots were developed using the ECL detection system in order to facilitate the detection of very low levels of GLUT 2 expression. Results indicated that GLUT 2 was expressed at higher levels in clone 3T3/G2a relative to clone 3T3/G2b since the intensity of the GLUT 2 band was greater for 3T3/G2a when equal amounts of lysate from both clones were immunoblotted. It was hoped that by utilising both clones in subsequent experiments it would be possible to control for the potential effects of GLUT 2 over-expression.

Figure 4.3

Immunological identification of GLUT 2 in GLUT 2-transfected 3T3-L1 fibroblast cell lysates



Cell lysates were prepared from a number of G418-resistant GLUT 2-transfected 3T3-L1 fibroblast clones. 5µl volumes of each sample (from a total volume of 200µl/35mm plate) were electrophoresed on 10% polyacrylamide gels and proteins were transferred to nitrocellulose membranes (see 2.3, 2.4). The nitrocellulose membranes were then probed with 10µg/ml affinity purified anti-GLUT 2 antibodies in 1% non fat milk/1st wash buffer, and the blots were developed using the ECL detection system. The positions of the molecular weight markers in kilodaltons are indicated to the left hand side of the immunoblot.

Samples contain 5µg rat liver membranes (lane 1), wild-type 3T3-L1 fibroblast lysate (lane 2), clone 3T3/ca lysate (lane 3), clone 3T3/cb lysate (lane 4), clone 3T3/G2b lysate (lane 5), clone 3T3/G2a lysate (lane 6), clone 3T3/G2a lysate (lane 7), clone 3T3/G2a lysate (lane 8).

4.3.3 [^{3}H] 2-deoxyglucose and [^{14}C] D-fructose uptake in transfected and non-transfected cells

Fibroblasts were grown to confluency on 6-well plates and [^{3}H] 2-deoxy-D-glucose or [^{14}C] D-fructose uptake was determined in resting cells and in cells stimulated for 30 min with 1μM insulin. Uptake values were corrected for any non-specific association of sugar with the cells by subtracting uptake values measured in the presence of 10μM cytochalasin B, a potent inhibitor of transport (2.10).

Assays were performed in triplicate for all incubation conditions and data from representative experiments for [^{3}H] 2-deoxyglucose and [^{14}C] D-fructose are shown in Figures 4.4 and 4.5, respectively, and in Table 4.2.

Results from figure 4.4 show that in resting cells 2-deoxyglucose uptake was 4.7 and 3.5 -fold higher in clones 3T3/G2a and 3T3/G2b, respectively, relative to wild-type fibroblasts, suggesting that GLUT 2 contributed significantly to basal 2-deoxyglucose transport.

Insulin stimulation resulted in 30% and 50% increases in 2-deoxyglucose uptake relative to basal levels in clones 3T3/G2a and 3T3/G2b respectively. A 2 -fold increase over basal uptake was observed in wild-type fibroblasts which is the typical response observed upon exposure to mitogens and probably represents translocation of GLUT 1 to the plasma membrane (Merrall *et al.*, 1993). These data suggest that although GLUT 2 contributed to increased basal 2-deoxyglucose uptake in GLUT 2 expressing clones, the protein did not appear to be insulin-responsive. It is likely that the small increase in 2-deoxyglucose uptake seen in clones 3T3/G2a and 3T3/G2b upon exposure to insulin represented increased uptake mediated by GLUT 1 translocation to the plasma membrane (see discussion).

In terms of the absolute amount of increase in 2-deoxyglucose uptake observed in response to insulin, results indicated that the increase was similar in clone 3T3/G2a, clone 3T3/G2b and wild-type cells (Fig.4.4 and Table 4.2a).

Therefore, on the basis of these data it could be suggested that this absolute amount of increase in response to insulin was mediated entirely by the GLUT 1 protein.

Data from Figure 4.5 shows that D-fructose uptake was approximately 9 -fold higher in clone 3T3/G2a and 2 -fold higher in clone 3T3/G2b relative to control-transfected cells. These results indicated that GLUT 2 was expressed at sufficiently high levels to function as a transporter of D-fructose, in addition to 2-deoxyglucose.

Upon insulin stimulation D-fructose was not significantly increased relative to basal levels in 3T3/G2a but, in clone 3T3/G2b, uptake was increased approximately 3-fold.

TABLE 4.2

The tables below show [^{3}H] 2-deoxyglucose uptake or [^{14}C] D-fructose uptake measured in GLUT 2-transfected, control-transfected and wild-type fibroblasts. 3T3-L1 fibroblast cells were grown to confluency on 6-well plates and then incubated in the absence or presence of 1μM insulin for 30 min. Sugar uptake was initiated with the addition of 50μl of [^{3}H] 2-deoxyglucose and [^{14}C] D-fructose, such that the final concentrations were 0.1mM and 1mM respectively and 0.5μCi/ml. Uptake was measured for 5 min. Non-specific sugar uptake was corrected for by subtracting uptake measured in the presence of 10μM cytochalasin B. Assays were performed in triplicate for all incubation conditions and data from representative experiments are shown below in pmols/million cells/min. A graphic representation of these data are shown in Figs.4.4 and 4.5.

(a) **[^{3}H] 2-DEOXYGLUCOSE UPTAKE** (n=3)

CLONE	BASAL	30' INSULIN	FOLD INCREASE
3T3/G2a	52 ± 11	68 ± 23	1.3
3T3/G2b	39 ± 8.5	62 ± 8.8	1.5
3T3/ca	5.4 ± 3.4	5.5 ± 1.2	-
WT	11 ± 0.45	23 ± 1.2	2.0

(b) **[^{14}C] D-FRUCTOSE UPTAKE** (n=3)

CLONE	BASAL	30' INSULIN	FOLD INCREASE
3T3/G2a	46.5 ± 2.2	49.5 ± 2.0	-
3T3/G2b	11.4 ± 2.3	33.5 ± 3.5	2.9
3T3/ca	3.8 ± 1.2	5.2 ± 5.2	1.3

FIGURE 4.4

Deglc transport in GLUT 2-transfected 3T3-L1 fibroblasts

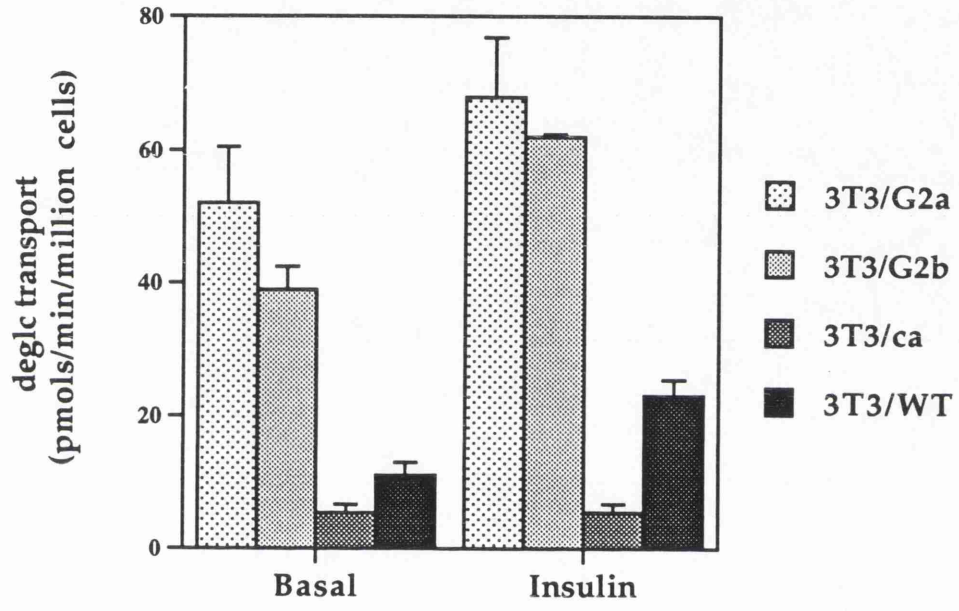
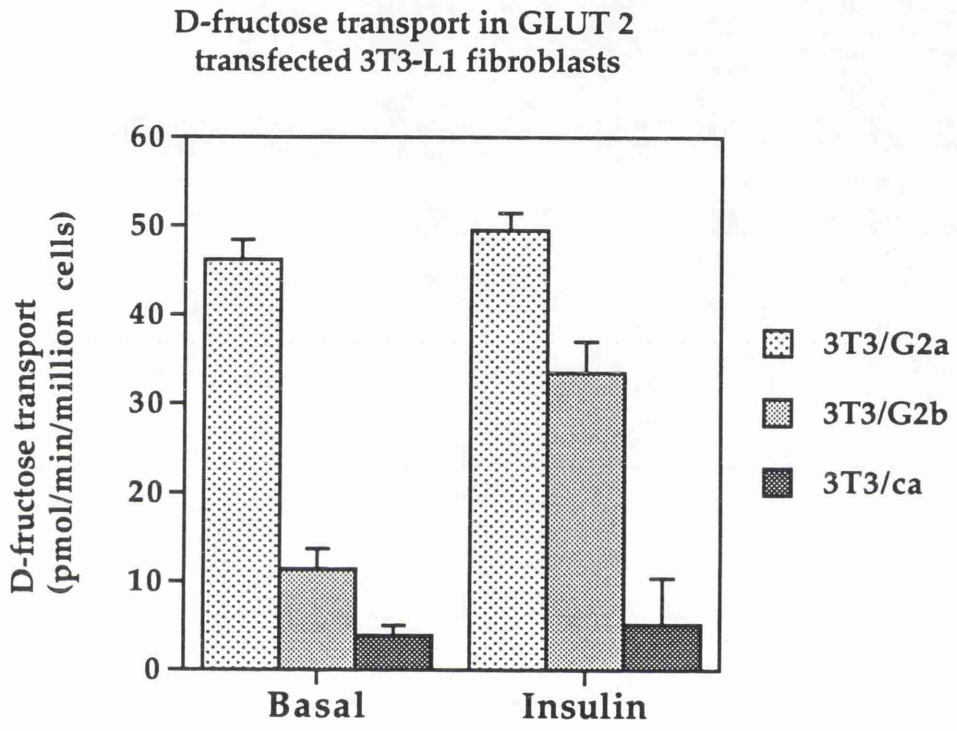


FIGURE 4.5


D-fructose transport in GLUT 2
transfected 3T3-L1 fibroblasts
D-fructose transport
(pmol/min/million cells)
60
50
40
30
20
10
0
Basal
Insulin
3T3/G2a
3T3/G2b
3T3/ca

4.4 DISCUSSION

Results from restriction digestion reactions (Table 4.1) carried out on a number of LK444/GLUT 2 DNA samples indicated that, out of 4 samples tested, samples I, O and 13 contained GLUT 2 cDNA in the correct orientation. The orientation of the insert was further confirmed after automated DNA sequencing of the 3 samples.

3T3-L1 fibroblasts were then transfected with the construct by lipofection and a number of G418-resistant clones were isolated and expanded. The data presented in Fig. 4.3 shows that GLUT 2 was detectable as 2 bands at 55kDa and 27.5 KDa in two clones designated 3T3/G2a and 3T3/G2b and that the protein was expressed at different levels in each clone. These two bands, detected by anti-GLUT 2 antibodies, probably represented 2 differentially glycosylated forms of GLUT 2 protein. It is quite possible however that the smaller of the two bands represented a GLUT 2-derived fragment or indeed incompletely translated GLUT 2 protein. Previous studies have been carried out in an attempt to de-glycosylate GLUT 2 protein in an effort to determine if the lower molecular weight GLUT 2 species represents unglycosylated GLUT 2. However, the protein could not be de-glycosylated using procedures which have proved effective in the deglycosylation of GLUTs 1, 3 and 4 (Brant *et al.*, unpublished data).

The uptake of 2-deoxyglucose and D-fructose was measured in clones 3T3/G2a and 3T3/G2b and the results were compared with uptake measured in non-transfected, wild-type 3T3-L1 cells and clone 3T3/ca cells which did not express GLUT 2 protein. The results for 2-deoxyglucose uptake revealed that basal uptake in clones 3T3/G2a and 3T3/G2b was significantly higher relative to both wild-type fibroblasts and clone 3T3/ca (Table 4.2, Fig.4.4). These results suggested that the increased uptake observed in GLUT 2 expressing clones was due to the expression of the transfected transporter. In addition, it could be postulated that GLUT 2 was localised at the plasma membrane since it contributed to the uptake of 2-deoxyglucose

under basal conditions. When cells were stimulated with insulin, uptake was increased by 16, 23 and 12 pmol/million cells/min in 3T3/G2a, 3T3/G2b and wild-type fibroblasts respectively (Table 4.2).

This insulin-stimulated increase in transport observed in all cells probably represented uptake mediated by GLUT 1 since the absolute amount of increase in 2-deoxyglucose uptake in all cells was not significantly different. It has previously been reported that 2-deoxyglucose uptake is stimulated approximately 2-fold in 3T3-L1 fibroblasts which results from the translocation of GLUT 1 from an intracellular location to the plasma membrane (Shibasaki *et al.*, 1992; Yang *et al.*, 1992). Therefore, it could be suggested from sugar uptake results that GLUT 2 is targeted in the transfected cell in a similar manner to that in its native cell i.e. at the plasma membrane. However, since subcellular fractions could not be prepared from fibroblasts and GLUT 2 localisation by immunofluorescence was not carried out the validity of these speculations could not be confirmed. Studies by other investigators have demonstrated that GLUT 1 and GLUT 4, when expressed in 3T3-L1 or CHO cells are localised to an intracellular region and GLUT 1 to the plasma membrane (Shibasaki *et al.*, 1992; Hudson *et al.*, 1992).

D-fructose uptake results showed that, under basal conditions, transport was higher in 3T3/G2a and in 3T3/G2b relative to the non-expressing 3T3/ca clone. Uptake was greater in 3T3/G2a which expressed higher levels of GLUT 2 than 3T3/G2b.

Upon insulin stimulation the fold increase in D-fructose uptake over basal was greatest in 3T3/G2b although very small increases, approximately 25% and 12.5%, were observed in 3T3/G2a and 3T3/ca respectively. It is unlikely that this insulin-stimulated increase in D-fructose uptake was mediated by GLUT 1 since this transporter does not transport D-fructose. Hence, it could be hypothesised that fibroblasts express an, as yet unidentified, D-fructose-specific transporter.

When cells were stimulated with insulin, uptake was increased by 3 and 2 pmols/million cells/min in 3T3/G2a and wild-type cells respectively, however, an

increase of 22 pmols/million cells/min was observed in clone 3T3/G2b. The larger increase observed in 3T3/G2b may have resulted from some degree of translocation of GLUT 2 to the plasma membrane in a similar manner to that seen for GLUT 1 insulin-stimulated 2-deoxyglucose uptake in 3T3-L1 fibroblasts. The increase observed upon insulin stimulation was lower in 3T3/G2a relative to clone 3T3/G2b. It may have been the case that since GLUT 2 expression was higher in 3T3/G2a relative to 3T3/G2b that any increase in transport due to GLUT 1 was not apparent above the GLUT 2-mediated uptake. In addition it could be speculated that the majority of transfected protein was expressed at the plasma membrane and hence intracellular protein was not available which could be recruited from intracellular stores. However, as mentioned previously, since immunolocalisation studies were not carried out, this hypothesis could not be confirmed .

Taken together, these results suggest that GLUT 2, when expressed in 3T3-L1 fibroblasts, does not translocate in response to insulin since D-fructose uptake was not significantly increased upon insulin exposure. However, it could be postulated, on the observation that the absolute amount of D-fructose uptake in response to insulin was greater in 3T3/G2b fibroblasts relative to that in 3T3/G2a and wild-type fibroblasts, that a proportion of GLUT 2 protein was intracellularly localised in 3T3/G2b cells which, in response to insulin, translocated to the plasma membrane in a similar manner to GLUT 1. This putative GLUT 2 translocation in 3T3/G2b cells may not have occurred in 3T3/G2a cells however, since total cell GLUT 2 protein levels in these latter cells were higher. Consequently, it could be postulated that more of the GLUT 2 protein was present at the plasma membrane in 3T3/G2a cells since the intracellular membrane capacity for GLUT 2 protein had been exceeded. Hence, GLUT 2 recruitment to the cell surface in response to insulin may not have been required in 3T3/G2a cells.

In 3T3-L1 adipocytes insulin stimulation results in a 20 to 30 -fold increase in 2-deoxyglucose uptake due to the translocation of GLUT 4 protein. Insulin stimulation of GLUT 2-transfected fibroblasts resulted in only a very small increase

(less than 2 fold) in 2-deoxyglucose uptake. Hence, even if translocation was occurring in GLUT 2-transfected cells the extent of translocation was insignificant when compared to the extent of GLUT 4 translocation observed in 3T3-L1 adipocytes.

It could also be hypothesised that GLUT 2 did not appear to be insulin-sensitive since the cell in which it was expressed is not itself highly responsive to insulin.

Studies on the expression of GLUT 2 and the effects of insulin must now be assessed when the cells have been differentiated into adipocytes, since in this situation GLUT 2 would be expressed in a cell environment where glucose transport would be highly insulin-regulated. The subcellular location of the expressed protein and its distribution in unstimulated and stimulated cells should be determined to assess whether its targeting is similar to that of the native fat cell transporter GLUT 4. Consequently, as a result of such investigations it should be possible to determine whether GLUT 2 acquires insulin-sensitivity and the characteristics of GLUT 4 by exhibiting translocation. These are the points which will be addressed in the following part of the study.

CHAPTER 5

THE EXPRESSION OF GLUT 2 IN 3T3-L1 ADIPOCYTES TO STUDY GLUT 2 LOCALISATION AND INSULIN-RESPONSIVENESS

5.1 INTRODUCTION

3T3-L1 fibroblasts, under defined conditions, can be induced to undergo differentiation to become adipocytes. This conversion is correlated with the accumulation of cytoplasmic triglycerides and the acquisition of morphological and biochemical properties characteristic of mature fat cells (Green, 1978). Concomitant with these morphological changes, the cells exhibit 10 to 100 -fold increases in *de novo* lipogenesis (Green and Kehinde, 1975; Mackall *et al.*, 1976), 40 to 50 -fold increases in the activities of ATP-citrate lyase, fatty acid synthetase and acetyl-CoA carboxylase (Mackall *et al.*, 1976). The cells also acquire the ability to synthesise cAMP in response to adrenocorticotrophic hormone (ACTH), become more sensitive to β-adrenergic-mediated stimulation of adenylate cyclase, and develop sensitivity to the metabolic effects of physiological concentrations of insulin (Rubin *et al.*, 1977). Although specific insulin receptors are present in cells before the onset of differentiation, the number of insulin receptors per cell increases 35 -fold during conversion to adipocytes (Rubin *et al.*, 1978).

In the fibroblastic form of the 3T3-L1 cell, GLUT 1 is the only glucose transporter isoform expressed (Kaestner *et al.*, 1989; deHerreros and Birnbaum, 1989). Upon differentiation however, GLUT 4 is expressed in addition to GLUT 1, with GLUT 4 mRNA and protein being detectable 3 days following the initiation of differentiation (deHerreros and Birnbaum, 1989). Moreover, the appearance of GLUT 4 in the cell correlates with the acquisition of an increased stimulation of glucose uptake in response to stimulation with maximal concentrations of the hormone (deHerreros and Birnbaum, 1989)

It is now well established that GLUT 4 is responsible for the rapid and large 20 to 30 -fold increase in glucose uptake observed when adipocytes are exposed to insulin. Numerous groups have shown independently, that insulin promotes the redistribution of GLUT 4 from an unidentified intracellular region to the plasma membrane by a process termed translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Slot *et al.*, 1991a). Subsequently, studies carried out in rat adipocytes and 3T3-L1 adipocytes have shown that, in resting cells, approximately 90% of GLUT 4 protein is located in an intracellular pool, and that GLUT 4 protein levels at the plasma membrane increase following insulin stimulation (Zorzano *et al*, 1989; Holman *et al.*, 1990; Calderhead *et al.*, 1990; Piper *et al.*, 1991; Slot *et al.*, 1991a). In addition to the 15 to 20 -fold increase in GLUT 4 protein at the plasma membrane, there is also a small 2 to 3 -fold increase in GLUT 1 protein (Holman *et al.*, 1990). Hence this increase in total transporter number at the cell surface accounts for most, if not all, of the increase in glucose transport observed in adipocytes in response to insulin. Thus in adipocytes, insulin increases the rate of glucose uptake by increasing the number of functional glucose transporters at the cell surface.

In contrast, insulin does not stimulate an increase in glucose uptake in the liver (Baur and Heldt, 1977; Williams *et al.*,1968) despite the presence of insulin receptors. Immunocytochemical studies have shown that GLUT 2 is expressed in all hepatocytes, where it is restricted to the sinusoidal plasma membrane, with high expression in the periportal area and lower expression in the perivenous area (Thorens *et al.*, 1990; Hacker *et al.*, 1991). The physiological role of glucose transport in the liver differs from that in most other tissues, since this organ can both take up and release glucose in order to maintain blood glucose levels. When blood glucose levels are high, glucose is transported into the liver and is converted to glycogen. When blood glucose levels are low, glycogen is broken down and glucose is once again released into the blood where it can be carried to other tissues for metabolism. The high K_m of GLUT 2 (15mM) (Colville *et al.*, 1993) may reflect a special adaptation for this role, allowing glucose flux into and out of the hepatocyte to

change linearly with corresponding changes in blood glucose concentrations. This property would thus ensure that glucose transport would never become rate-limiting for either metabolism or supply to the blood.

As mentioned previously it has been demonstrated that GLUT 2 is localised to the plasma membrane of hepatocyte cells and is absent from intracellular hepatocyte membranes (Thorens *et al.*, 1990). Taken together with the observation that insulin does not promote increased uptake of glucose in the liver, these data suggest that GLUT 2 does not exhibit insulin-induced translocation.

This study was carried out in an attempt to determine whether GLUT 2, when expressed in an insulin-sensitive fat cell environment, would be targeted to the plasma membrane, as in its native cell, or if it would be targeted to an intracellular region like the native adipocyte transporter GLUT 4. In addition, the possibility that insulin could promote the translocation of GLUT 2 in the adipocyte environment was investigated.

It could be hypothesised that, even if GLUT 2 was intracellularly targeted within the fat cell, the protein would not be localised to a specific vesicle population which could be induced by insulin to undergo translocation. The results of this study should determine the subcellular location of GLUT 2, its sensitivity to insulin and consequently whether cell environment and/or glucose transporter sequence dictates the targeting and the translocation process.

5.2 METHODS

5.2.1 Subcellular fractionation of 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated at 37°C in serum-free DMEM medium for 2-3 hours and then for a further 30 min, in the presence or absence of 1μM insulin, prior to subcellular fraction.

Plasma membrane and low density microsomal fractions were then prepared by the method of Piper *et al.* (1991) (see 2.9.2).

Protein concentrations were determined for each membrane fraction using the method of Peterson (1977) (see 2.2.2) and proteins were resuspended at 1-3mg/ml in 1X SDS Laemmli sample buffer containing 20mM DTT.

5.2.2 Preparation of total cell membranes for quantitation of GLUT 4 protein/mg of total protein

GLUT 2-transfected and wild-type adipocytes were grown on 10 cm plates and maintained in 20% and 10% foetal calf serum/DMEM medium, respectively. 2-3h prior to membrane preparation, cells were incubated in serum-free DMEM medium, following which, the medium was aspirated and the cells washed 3 times in ice-cold PBS, pH 6.5. The cells were then scraped, using a plastic cell scraper, into 2ml/plate of ice-cold PBS containing protease inhibitors (1μg pepstatin A, 0.2mM DFP, and 4μM Ep 64). Cells were homogenised with 20 handstrokes in a Dounce homogeniser and centrifuged at 180,000 x g for 1 hour. The supernatants were then carefully decanted and pellets were resuspended in a suitable volume of PBS containing protease inhibitors.

Protein concentrations were determined by the method of Peterson (1977) (see 2.2.2) and the samples were resuspended at 1-2mg/ml in 1 x SDS Laemmli buffer.

Membrane samples were then subjected to electrophoresis on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. Membranes were immunoblotted with anti-GLUT 4 antibodies (see 2.5.1 and 2.6.1), and quantitation of GLUT 4 immunoblot signals was carried out by carefully excising the appropriate regions of the nitrocellulose and counting in a γ-counter.

5.2.3 Immunoblotting of subcellular membrane fractions

Plasma membrane and low density microsomal fraction samples were electrophoresed on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. Membranes were then blocked in 5% non-fat milk/1st wash buffer for 30 min, and probed with anti-GLUT 2 or anti-GLUT 4 antibodies at 10μg/ml in 1% non-fat milk/1st wash buffer (see 2.5.1). Nitrocellulose membranes probed with anti-GLUT 4 antibodies were developed using ^{125}I-labelled goat anti-rabbit IgG, whereas those probed with anti-GLUT 2 antibodies were developed using the ECL detection system. The ECL system was used to detect GLUT 2 immunoblot signals since this system facilitated the detection of very low amounts of protein.

Quantitation of GLUT 4 was carried out as described previously and levels of GLUT 4 in membrane fractions were expressed as a percentage of the amount of GLUT 4 present in total cell membranes.

GLUT 2 signals in membrane fractions were quantitated by densitometric scanning and were expressed as a percentage of the total amount of GLUT 2 contained in total cell membranes. GLUT 2 levels could not be expressed as ng GLUT 2/μg total membrane protein since no standards were available, in which the absolute amount of this transporter expressed per μg of total protein was known.

5.2.4 Uptake of 2-deoxyglucose and D-fructose in GLUT 2-transfected and non-transfected adipocytes

3T3-L1 fibroblasts were grown to confluency on collagen coated 6-well plates in 10% calf serum/DMEM medium. (Cell culture plasticware was coated with a thin layer of collagen to promote cell adhesion. Previous studies by Gould *et al.* (1989), using transfected adipocytes, indicated that cells were prone to "lifting" off culture dishes shortly after differentiation (see 2.8.9)).

2 days post confluence cells were differentiated in 10% foetal calf serum/DMEM containing 0.5mM methyl isobutylxanthine, 0.25μM dexamethasone, and 1μg/ml insulin (see 2.8.5).

Adipocytes were assayed for [^{3}H] 2-deoxyglucose and [^{14}C] D-fructose uptake 8-13 days post-differentiation since at this time hexose transport was maximal.

2 hours prior to assay, cells were incubated in serum-free DMEM medium after which time the uptake of 0.1mM 2-deoxyglucose or 1mM D-fructose was measured for 5 min (see 2.10). Uptake was assessed after incubating the cells for 30 min. in the absence or presence of 1μM insulin. In addition, non-specific sugar uptake was corrected for by subtracting the uptake values obtained when the assays were performed in the presence of 10μM cytochalasin B, a potent inhibitor of transport.

5.2.5 Uptake of 2-deoxyglucose and D-fructose after pre-treatment overnight with inhibitors of the hexosamine pathway

GLUT 2-transfected and wild-type fibroblasts were grown on 6-well plates in 20% or 10% calf serum/DMEM medium, respectively, and 2 days post-confluence cells were differentiated (see 2.8.4 and 2.8.5). 10 days post differentiation, DMEM growth medium was aspirated and replaced with growth medium containing 0-

diaoacetyl-L-serine (azaserine) or 6-diazo-5-oxonorleucine at final concentrations of 30, 75, and150μM. The cells were then incubated overnight at 37°C after which time the uptake of 2-deoxyglucose was assessed. 2-deoxyglucose uptake was measured both in the absence and presence, for 30 min, of 1μM insulin (see 2.10).

5.3 RESULTS

5.3.1 GLUT 2 and GLUT 4 expression in adipocyte subcellular membrane fractions

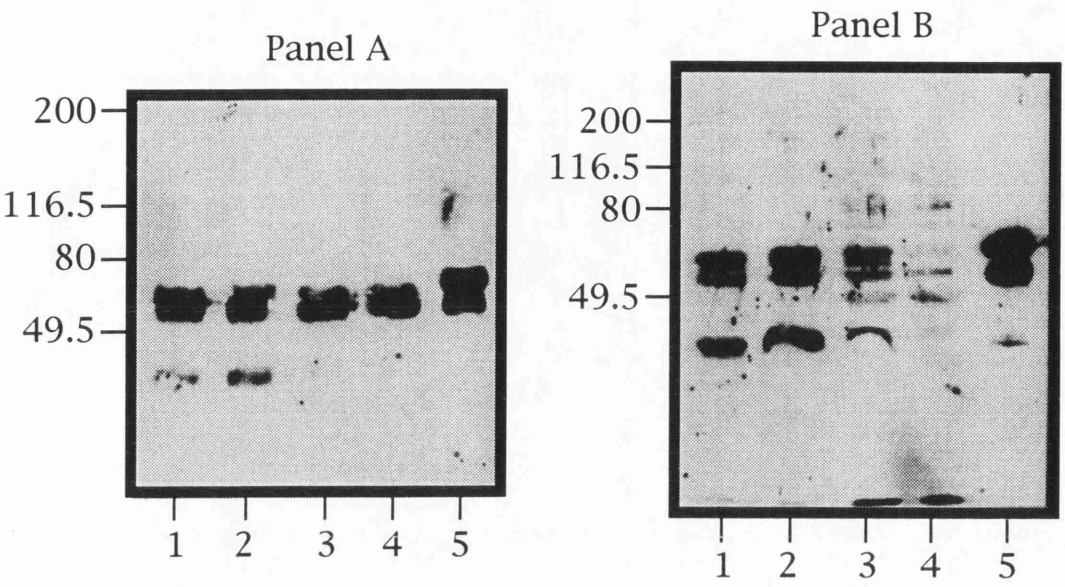
Plasma membrane (PM) and low density microsomal (LDM) fractions were prepared from basal and insulin-stimulated GLUT 2-transfected and non-transfected 3T3-L1 adipocytes. The samples were then subjected to electrophoresis and immunoblotting with affinity purified anti-GLUT 4 and anti-GLUT 2 antibodies (see 2.5.1).

Immunoblotting of samples prepared from GLUT 2 expressing clones, 3T3/G2a and 3T3/G2b, with anti-GLUT 2 antibodies (Fig. 5.1a and b) revealed that 3 bands were detectable at 63, 54, and 31kilodaltons in plasma membranes and low density microsomal fractions prepared from basal and insulin-stimulated cells (for explanation regarding the immunoreactivity of multiple bands with anti-GLUT 2 antibodies see 4.3.2). Immunoreactive bands at similar molecular weights were detected also in membranes prepared from rat liver which indicated that the immunoreactive bands detected in adipocyte membrane fractions represented GLUT 2 protein.

The levels of GLUT 2 detected in plasma membrane samples prepared from basal and insulin-stimulated 3T3/G2a cells appeared visually to be of equal intensity, when equal amounts of the samples were probed. This suggested that the expressed GLUT 2 protein did not undergo translocation in response to insulin. Densitometric scanning of these immunoreactive bands in plasma membrane samples prepared from basal and insulin-stimulated 3T3/G2a cells demonstrated also that no significant increase in GLUT 2 signal was observed in the fraction prepared from insulin stimulated cells relative to that prepared from basal cells (see Table 5.1a). This confirmed that the expressed GLUT 2 protein did not undergo translocation from the

FIGURE 5.1

Immunological identification of GLUT 2 in plasma membrane (PM) and low density microsome (LDM) fractions prepared from basal (bas) and insulin-stimulated (ins) adipocytes



Subcellular membrane fractions were prepared from 3T3/G2a (panel a) and 3T3/G2b (panel b) adipocytes (see 2.9.2). Samples were electrophoresed on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. Nitrocellulose membranes were then probed with 10µg/ml anti-GLUT 2 antibodies in 1% non-fat milk/1st wash buffer and blots were developed using the ECL detection system (see 2.6.2).

Panel a: 3T3/G2a samples contain 10µg bas PM (lane 1), 8µg ins PM (lane 2), 28µg bas LDM (lane 3), 24µg ins LDM (lane 4), 5µg rat liver membranes (lane 5).

Panel b: 3T3/G2b samples contain 23µg bas PM (lane 1), 23µg ins PM (lane 2), 30µg bas LDM (lane 3), 37µg ins LDM (lane 4), 5µg rat liver membranes (lane 5).

The entire running gel is shown and the positions of the molecular weight markers in kilodaltons are shown to the left of the immunoblot.

TABLE 5.1

Plasma membrane and low density microsomal fractions were prepared from 3T3/G2a and 3T3/G2b adipocytes incubated in the absence or presence, for 30 min, of 1μM insulin.

Membrane samples were electrophoresed and proteins were transferred to nitrocellulose membranes. Membranes were then probed with anti-GLUT 2 antibodies and blots were developed using the ECL detection system.

Tables 5.1a and 5.1b below show the levels of GLUT 2 protein per membrane fraction expressed as a percentage of GLUT 2 levels in total membranes. Quantitation of GLUT 2 immunoblot signals in membrane fractions was then carried out by scanning densitometry.

(a)

CLONE 3T3/G2a membrane fraction	% of total GLUT 2
PM basal	61
PM insulin	48
LDM basal	39
LDM insulin	52

(b)

CLONE 3T3/G2b membrane fraction	% of total GLUT 2
PM basal	55
PM insulin	85
LDM basal	45
LDM insulin	15

LDM to the plasma membrane upon exposure to insulin. GLUT 2 was also detectable in LDM fractions prepared from basal and insulin-treated cells but was present at slightly lower levels relative to those observed in plasma membrane fractions (see Table 5.1a). Furthermore, no reduction in GLUT 2 levels were observed in the LDM fraction prepared from insulin-stimulated cells relative to levels observed in basal cells which again suggested that GLUT 2 did not undergo translocation in response to insulin. Results in Table 5.1a show that a greater percentage of GLUT 2 resided in the plasma membrane fraction in basal cells. The percentage of GLUT 2 associated with the plasma membrane was not significantly changed in plasma membrane fractions prepared from insulin-treated cells relative to plasma membrane fractions prepared from basal cells. A lower percentage of GLUT 2 was expressed in the low density microsomes relative to the percentage expressed in the plasma membrane which indicated that the protein was predominantly plasma membrane-localised. It could be postulated that GLUT 2 protein, detected in the LDM fraction, represented a portion of GLUT 2 protein which was undergoing processing or indeed that it represented GLUT 2 which was located in intracellular membranes.

The levels of GLUT 2 detected in plasma membrane samples prepared from basal and insulin-stimulated 3T3/G2b cells appeared visually to be of greater intensity in fractions prepared from insulin-stimulated cells when equal amounts of the samples were probed (Table 5.1b). This suggested that the expressed GLUT 2 protein exhibited some degree of translocation in response to insulin.

Densitometric scanning of the immunoreactive bands in plasma membrane samples prepared from 3T3/G2b cells demonstrated that the levels of GLUT 2 protein were 30% greater in fractions prepared from insulin-stimulated cells relative to fractions prepared from basal cells (see Table 5.1b). GLUT 2 protein was also detectable in LDM fractions prepared from basal and insulin-treated cells but was present at lower levels relative to those observed in plasma membrane fractions (see Table 5.1a). However, the percentage of GLUT 2 protein associated with low density microsomes was lower in fractions prepared from insulin-stimulated cells

relative to fractions prepared from basal cells. Hence these data suggested that, in 3T3/G2b adipocytes, GLUT 2 exhibited insulin-induced translocation from low density microsomes to the plasma membrane resulting in an increase in GLUT 2 protein at the plasma membrane and a concomitant decrease in low density microsomal fractions.

As expected, no GLUT 2 expression was detectable in subcellular membrane fractions prepared from wild-type adipocytes or 3T3/ca control-transfected adipocytes (data not shown).

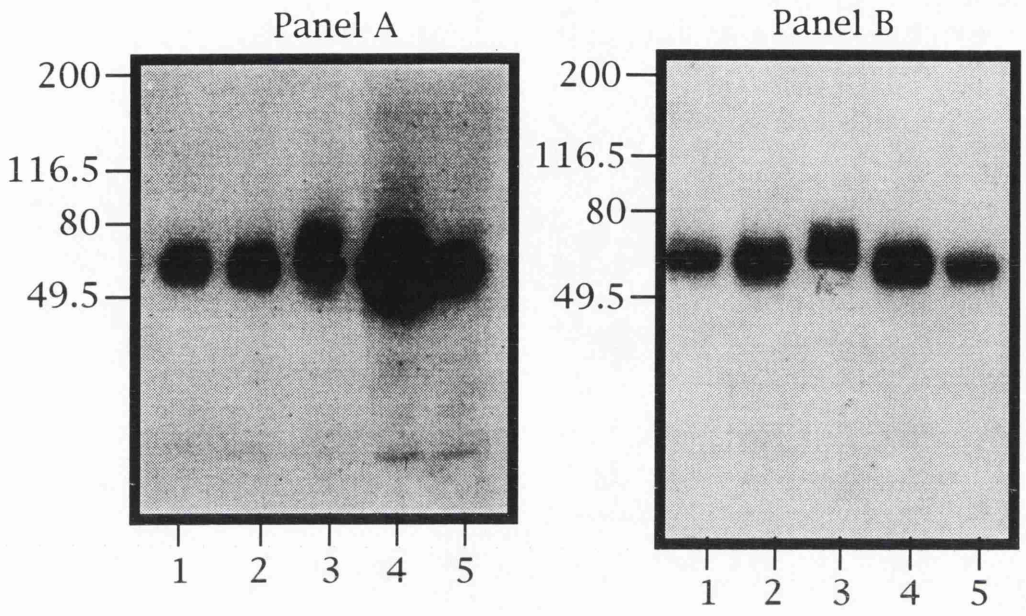
Immunoblotting of membrane fractions with anti-GLUT 4 antibodies revealed that a band was detectable at 50kDa in both PM and LDM fractions prepared from 3T3/G2a, 3T3/G2b, and wild-type adipocytes (Fig. 5.2a, b and c). In all cases, the intensity of the GLUT 4 immunoreactive band was higher in PM fractions and lower in LDM fractions prepared from insulin-treated cells relative to the intensity observed in respective fractions prepared from basal cells (see Tables 5.2a, b and c). In basal cells the majority of GLUT 4 was detected in the low density microsomal fraction with very low levels being detected at the plasma membrane.

When levels of GLUT 4 protein were expressed as a percentage of total GLUT 4 protein present in total cell membranes (Tables 5.2a, b and c), results indicated that in clones 3T3/G2a, 3T3/G2b and in wild-type cells the majority of GLUT 4 (70 to 90%) was expressed in the LDM fraction in basal cells. A greater percentage of GLUT 4 was present in PM fractions prepared from insulin-stimulated cells relative to the percentage associated with basal plasma membranes. In addition there was a concomitant decrease in the percentage of GLUT 4 associated with low density microsomes prepared from insulin stimulated cells relative to LDM fractions prepared from basal cells. Hence GLUT 4 translocation appeared to occur within a similar range in wild-type adipocytes and GLUT 2-expressing 3T3/G2a and 3T3/G2b adipocytes.

Quantitation of GLUT 4 levels, by γ-counting in subcellular fractions indicated that in all clones GLUT 4 levels were increased 1.8-2.2 fold in the PM and

FIGURE 5.2

Immunological identification of GLUT 4 in plasma membrane (PM) and low density microsomal (LDM) fractions prepared from basal (bas) and insulin-stimulated (ins) 3T3-L1 adipocytes



Membranes were electrophoresed on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes (2.3 and 2.4). Immunoblots were then probed with 10μg/ml anti-GLUT 4 antibodies in 1% non-fat milk/1st wash buffer. Blots were then developed using ^{125}I goat anti-rabbit secondary antibody and autoradiography (see 2.6.1).

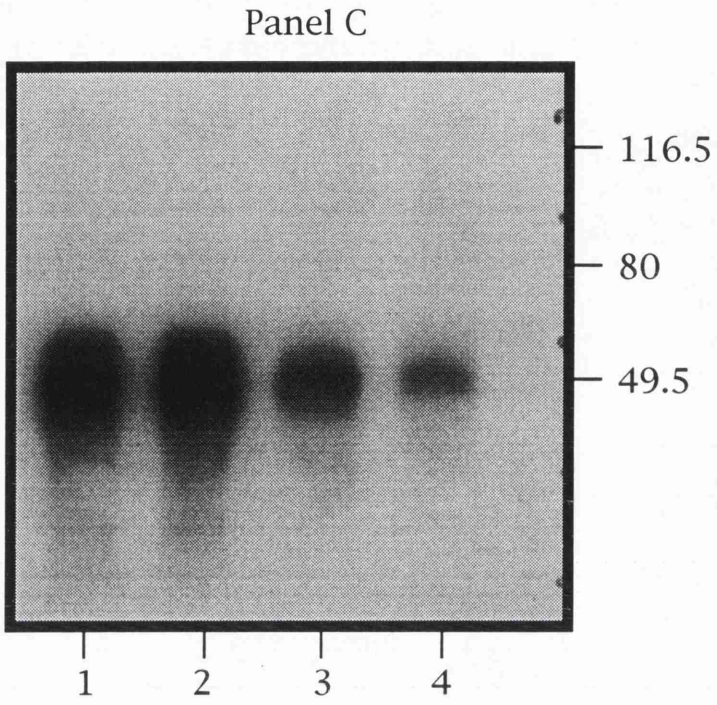
Panel a : Identification of GLUT 4 in clone 3T3/G2a subcellular fractions. Samples contain 12μg bas PM (lane 1), 6μg ins PM (lane 2), 10ng GLUT 4 standards (lane 3), 24μg bas LDM (lane 4), 13μg ins LDM (lane 5).

Panel b : Identification of GLUT 4 in clone 3T3/G2b subcellular fractions. Samples contain 23μg bas PM (lane 1), 23μg ins PM (lane 2), 10ng GLUT 4 standards (lane 3), 10μg bas LDM (lane 4), 12μg ins LDM (lane 5).

The positions of the molecular weight markers in kilodaltons are shown to the left hand side of the immunoblots.

FIGURE 5.2

Immunological identification of GLUT 4 in plasma membranes (PM) and low density microsomal (LDM) fractions prepared from basal (bas) and insulin-stimulated (ins) 3T3-L adipocytes



Panel c: Identification of GLUT 4 in wild-type 3T3-L1 adipocyte subcellular membrane fractions. Samples contain 20μg ins LDM (lane 1), 20μg bas LDM (lane 2), 20μg ins PM (lane 3), 20μg bas PM (lane 4).

The positions of the molecular weight markers in kilodaltons are indicated to the right hand side of the immunoblot.

TABLE 5.2

Tables 5.2a, 5.2b and 5.2c below show the levels of GLUT 4 protein per membrane fraction expressed as a percentage of GLUT 4 levels in total membranes. Quantitation of GLUT 4 immunoblot signals in membrane fractions was carried out by carefully excising the appropriate regions of the nitrocellulose and counting in a gamma counter.

(a)

CLONE 3T3/G2a membrane fraction	% of total GLUT 4
PM basal	11
PM insulin	56
LDM basal	89
LDM insulin	44

(b)

CLONE 3T3/G2b membrane fraction	% of GLUT 4
PM basal	26
PM insulin	53
LDM basal	74
LDM insulin	47

(c)

WILD TYPE 3T3-L1 membrane fraction	% of GLUT 4
PM basal	12
PM insulin	30
LDM basal	88
LDM insulin	70

decreased 1.35-1.8 fold in the LDM upon insulin stimulation (data not shown). Thus from these data it appeared that GLUT 4 translocation occurred to the same extent in transfected and non-transfected cells. These results were similar to those of Zorzano *et al.* who demonstrated that the increase in GLUT 4 protein at the plasma membrane fraction and decrease in the low density microsomal fraction were less than could account for the increase in 2-deoxyglucose uptake in response to insulin (Zorzano *et al.*, 1989) (see Chapter 1 for information regarding methods available to assess GLUT 4 translocation).

5.3.2 [^{3}H] 2-deoxyglucose and [^{14}C] D-fructose uptake in transfected and non-transfected adipocytes

[^{3}H] 2-deoxyglucose uptake rates were measured in 3T3/G2a and 3T3/G2b cells in the absence or presence, for 30 min, of 1μM insulin. Transport rates were then compared with those measured in wild-type adipocytes. Non-specific association of sugar with the cells was corrected for by subtracting uptake rates measured in the presence of cytochalasin B.

Assays were carried out in triplicate for each incubation condition and were performed at least 3 times for each clone. Transport rates from a representative experiment are shown in Table 5.3 and Fig. 5.3.

Results indicated that the basal uptake observed in 3T3/G2a and 3T3/G2b adipocytes was higher relative to uptake measured in wild-type adipocytes. These data suggested that the larger basal uptake rates observed in 3T3/G2a and 3T3/G2b were attributable to GLUT 2 protein, the majority of which was expressed at the plasma membrane.

These data, showing that basal uptake in GLUT 2 expressing clones was elevated relative to wild-type adipocytes, was as expected since the majority of native GLUT 4 transporter in the basal wild-type adipocyte cells would be intracellularly

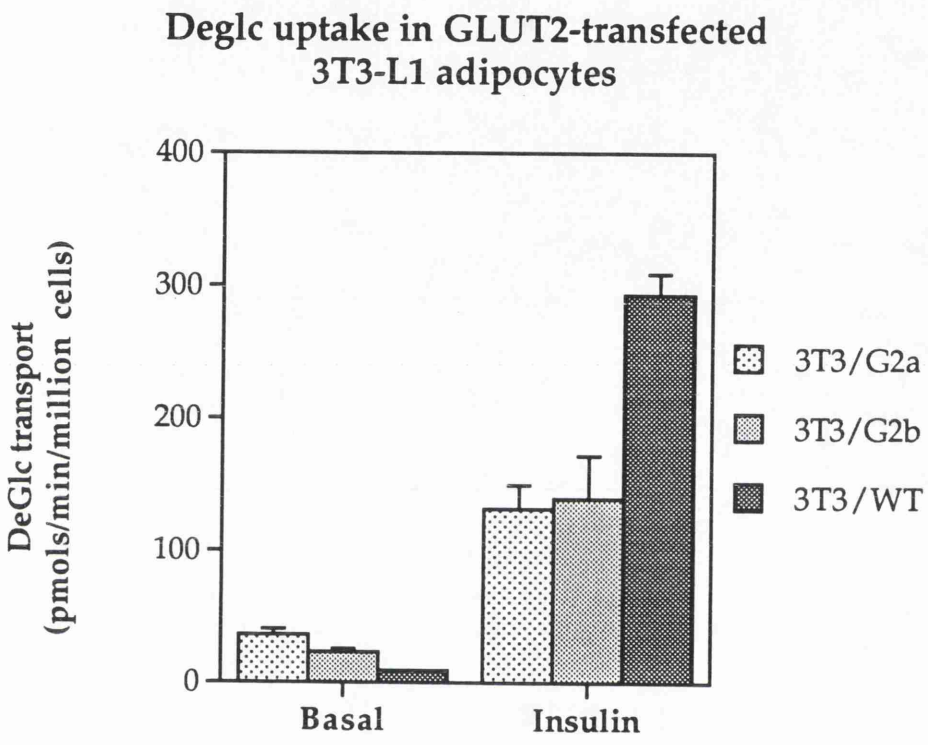
TABLE 5.3

2-DEOXYGLUCOSE UPTAKE IN GLUT 2-TRANSFECTED ADIPOCYTES AND WILD-TYPE ADIPOCYTES

The table below shows [^{3}H] 2-deoxyglucose uptake measured in wild-type and GLUT 2-expressing adipocytes. Cells, grown on 6-well plates were incubated in the absence or presence of 1μM insulin for 30 min. Glucose uptake was initiated by the addition of 0.1mM, 0.5μCi/ml [^{3}H] 2-deoxyglucose, (final concentrations) and uptake was measured for 5 min. Non-specific [^{3}H] 2-deoxyglucose uptake was corrected for by subtracting uptake measured in the presence of 10μM cytochalasin B. The data presented below, expressed in pmol/million cells/min is from a representative experiment. A graphic representation of this data is shown in Fig. 5.3.

CLONE	BASAL	30 min Ins	Mean fold increase
3T3/G2a	36 ± 4.3	132 ± 18	3 ± 0.6
3T3/G2b	23 ± 2.4	140 ± 32	9.6 ± 1.9
WILD TYPE	9.3 ± 1.5	293 ±16	20-30

FIGURE 5.3


Deglc uptake in GLUT2-transfected
3T3-L1 adipocytes
DeGlc transport
(pmols/min/million cells)
400
300
200
100
0
Basal
Insulin
3T3/G2a
3T3/G2b
3T3/WT

localised and would not be available at the plasma membrane to contribute to basal glucose transport.

Upon insulin stimulation 2-deoxyglucose transport rates were increased 3 -fold and 9 -fold over basal in clones 3T3/G2a and 3T3/G2b respectively. This was in marked contrast to the insulin-stimulated rates observed in wild type adipocytes which were 20 to 30 -fold higher than basal transport rates. Hence it appeared that GLUT 2-expressing clones had acquired a reduced sensitivity to insulin (see discussion).

When wild-type cells were assessed for their ability to transport [^{14}C] D-fructose in the absence and presence of 1μM insulin, it was observed that [^{14}C] D-fructose uptake rates were similar when measured in the absence and presence of cytochalasin B. Thus D-fructose uptake was not reduced in the presence of the transporter inhibitor. These observations suggested that a D-fructose transporter was expressed in these cells but that it was insensitive to the transport-inhibitory effects of cytochalasin B.

A marked reduction in [^{14}C] D-fructose uptake was observed in the presence of cytochalasin B in clone 3T3/G2a which expressed GLUT 2 protein at high levels. In contrast to these results however no cytochalasin B-inhibitable D-fructose transport was observed in GLUT 2-expressing 3T3/G2b adipocytes. This was probably due to these cells expressing only low levels of the cytochalasin B-inhibitable GLUT 2 D-fructose transporter. Recently Shepherd *et al.* demonstrated that, in addition to GLUTs 1 and 4, GLUT 5 is also expressed in the plasma membranes of human adipocytes (Shepherd *et al.*, 1992). Burant *et al.* demonstrated, by expressing GLUT 5 cDNA in *Xenopus* oocytes, that the protein showed little ability to transport 2-deoxyglucose but transported D-fructose with a K_m of 6mM. These investigators consequently suggested that this transporter functions primarily as a transporter of D-fructose. In addition it was shown in Burant's study that GLUT 5 mediated D-fructose uptake was insensitive to even 100μM cytochalasin B which is

a known inhibitor for other GLUT isoforms at concentrations even 10-fold lower (Burant *et al.*, 1992).

If this evidence is correct, it could be suggested that in the study presented here, cytochalasin B-sensitive D-fructose uptake would only be apparent in 3T3-L1 adipocyte cells in which a cytochalasin-B inhibitable fructose transporter was expressed. In this study, cytochalasin B inhibitable D-fructose uptake was observed only in clone 3T3/G2a which expressed the fructose transporter GLUT 2, at high levels. Cytochalasin B-inhibitable D-fructose uptake was not observed in 3T3/G2b cells which expressed GLUT 2 at low levels. Hence it could be suggested that any cytochalasin B-inhibitable GLUT 2 D-fructose transport potentially occurring in 3T3/G2b cells was "overridden" by cytochalasin B-insensitive GLUT 5-mediated D-fructose uptake.

It would have been informative to have assessed the level of GLUT 5 D-fructose transporter in both transfected and non-transfected 3T3-L1 adipocyte cells in this study, however anti-GLUT 5 antibodies were unavailable to carry out the required investigations.

Results from Table 5.4 and Fig. 5.4 show that basal D-fructose uptake in resting 3T3/G2a adipocyte cells was 44 pmols/million cells/min. Uptake was increased by only 2.2-fold after cells were stimulated with insulin for 30 min.

In wild-type adipocyte cells insulin stimulation resulted in the translocation of GLUT 4 protein and a concomitant 20 to 30 -fold increase in 2-deoxyglucose transport. Thus, it would be expected that if GLUT 2 translocated in response to insulin then a comparable 20 to 30 -fold increase in D-fructose would be observed in GLUT 2-expressing cells. Hence, since D-fructose uptake in 3T3/G2a was not increased by the same magnitude as 2-deoxyglucose uptake in wild-type cells in response to insulin, it would appear that, unlike GLUT 4, GLUT 2 does not undergo insulin-induced translocation.

TABLE 5.4

D-FRUCTOSE UPTAKE IN GLUT 2-TRANSFECTED ADIPOCYTES

The table below shows D-fructose uptake measured in GLUT 2-expressing adipocytes. Cells, grown on 6-well plates were incubated in the absence or presence of 1μM insulin for 30 min. Uptake was inititated by the addition of 1mM, 0.5μCi/ml [^{14}C] D-fructose (final concentrations) and uptake was measured for 5 min. Non-specific [^{14}C] D-fructose uptake was corrected for by subtracting uptake measured in the presence of 10μM cytochalasin B. The data represented below, expressed in pmol/million cells/min is from a representative experiment. A graphic representation of this data is shown in Fig. 5.4.

CLONE	BASAL	30 min Ins	Mean fold increase
3T3/G2a	44 ± 6.4	75 ± 14	2.2 ± 0.6

Cytochalasin B-inhibitable D-fructose uptake in wild type cells and in 3T3/G2b could not be demonstrated (see results and discussion).

FIGURE 5.4

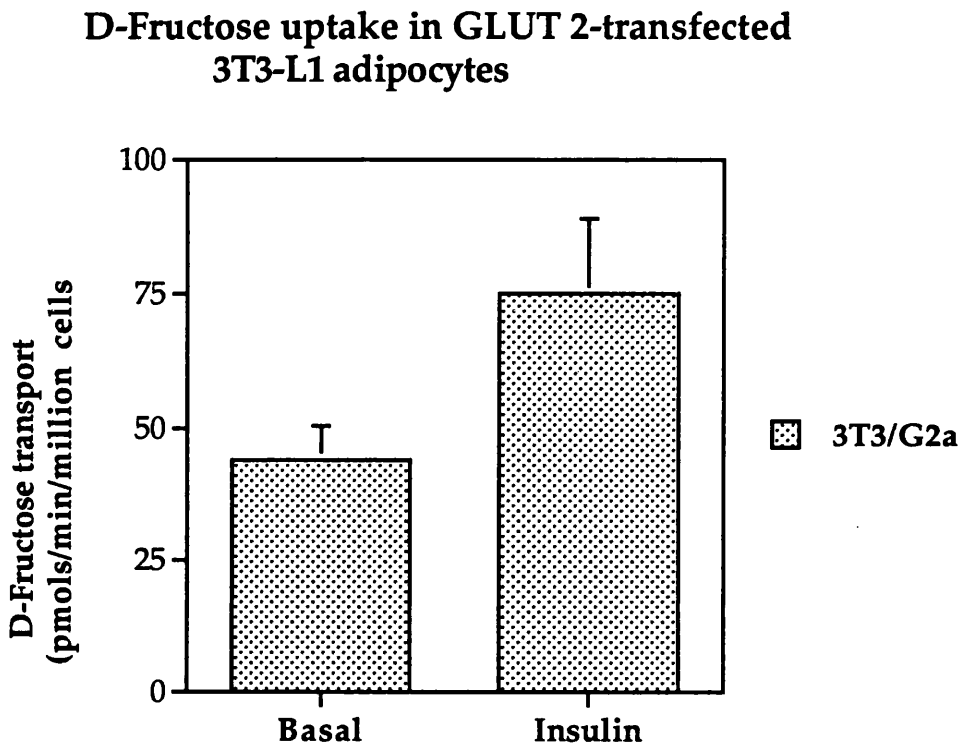
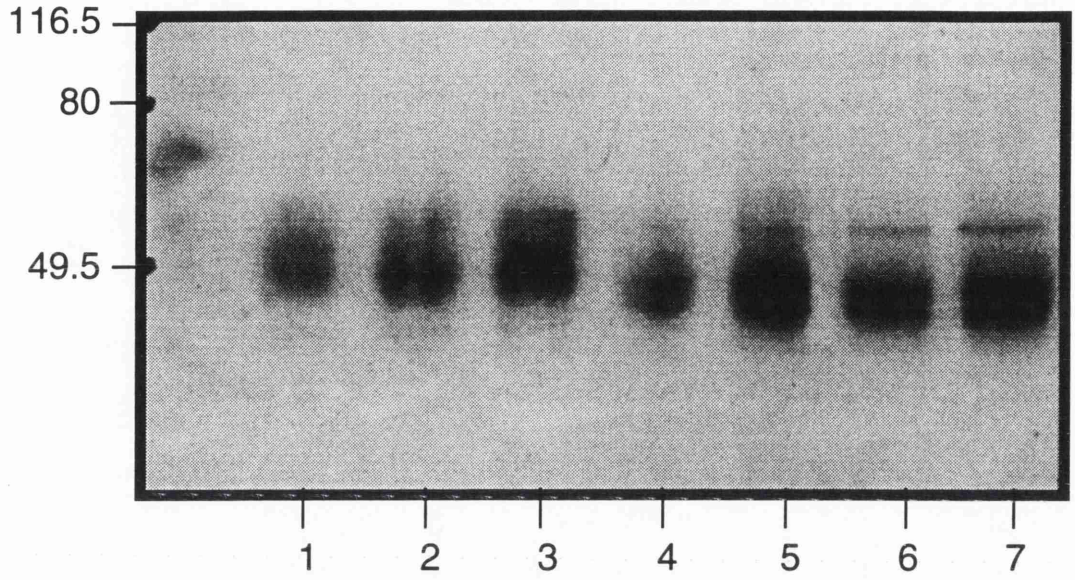

D-Fructose uptake in GLUT 2-transfected 3T3-L1 adipocytes
D-Fructose transport (pmols/min/million cells)
100
75
50
25
0
Basal
Insulin
3T3/G2a

FIGURE 5.5

Immunological identification of GLUT 4 in total cell membranes prepared from 3T3/G2a and wild-type 3T3-L1 adipocytes.



Total cell membranes were prepared from 3T3/G2a and wild-type adipocytes as described previously (see 5.2.2). The samples were electrophoresed and proteins were transferred to nitrocellulose membranes. The nitrocellulose membranes were then immunoblotted with 10μg/ml affinity purified anti-GLUT 4 antibodies. Quantitation of GLUT 4 was carried out by excising the appropriate regions of the nitrocellulose and counting in a γ-counter. The positions of the molecular weight markers are shown to the left hand side of the immunoblot.

Samples contain 5ng GLUT 4 (lane 1), 9ng GLUT 4 (lane 2), 12ng GLUT 4 (lane 3), 15μg wild-type membranes (lane 4), 20μg wild-type membranes (lane 5), 15μg 3T3/G2a membranes (lane 6), 20μg 3T3/G2a membranes (lane 7)

TABLE 5.5

GLUT-2 transfected (Tables 5.5a and 5.5b) or wild-type (Tables 5.5c and 5.5d) 3T3-L1 adipocytes were incubated overnight in growth medium containing 0μM, 30μM, 75μM, or 100μM of 0-diazoacetyl-L-serine or 6-diazo-5-oxonorleucine. The following day [^{3}H] 2-deoxyglucose uptake was assessed in the absence and presence, for 30 min, of 1μM insulin. The results, expressed as pmol/million cells/min are presented below.

(a)

O-diazoacetyl-L-serine concentration (μM)	Basal	30 min Insulin	Fold increase
0	34 ± 4.5	126 ± 15	3.7
30	34 ± 4	122 ± 9	3.5
75	33 ± 3.5	123 ± 4.5	3.7
150	34 ± 5	131 ± 12	3.8

(b)

6-Diazo-5-oxonorleucine concentration (μM)	Basal	30 min Insulin	Fold increase
0	34 ± 4.5	126 ± 15	3.7
30	35 ± 9	109 ± 20	3.1
75	31 ± 7	124 ± 4.5	4.0
150	33 ± 8	129 ± 12	3.9

TABLE 5.5 (c)

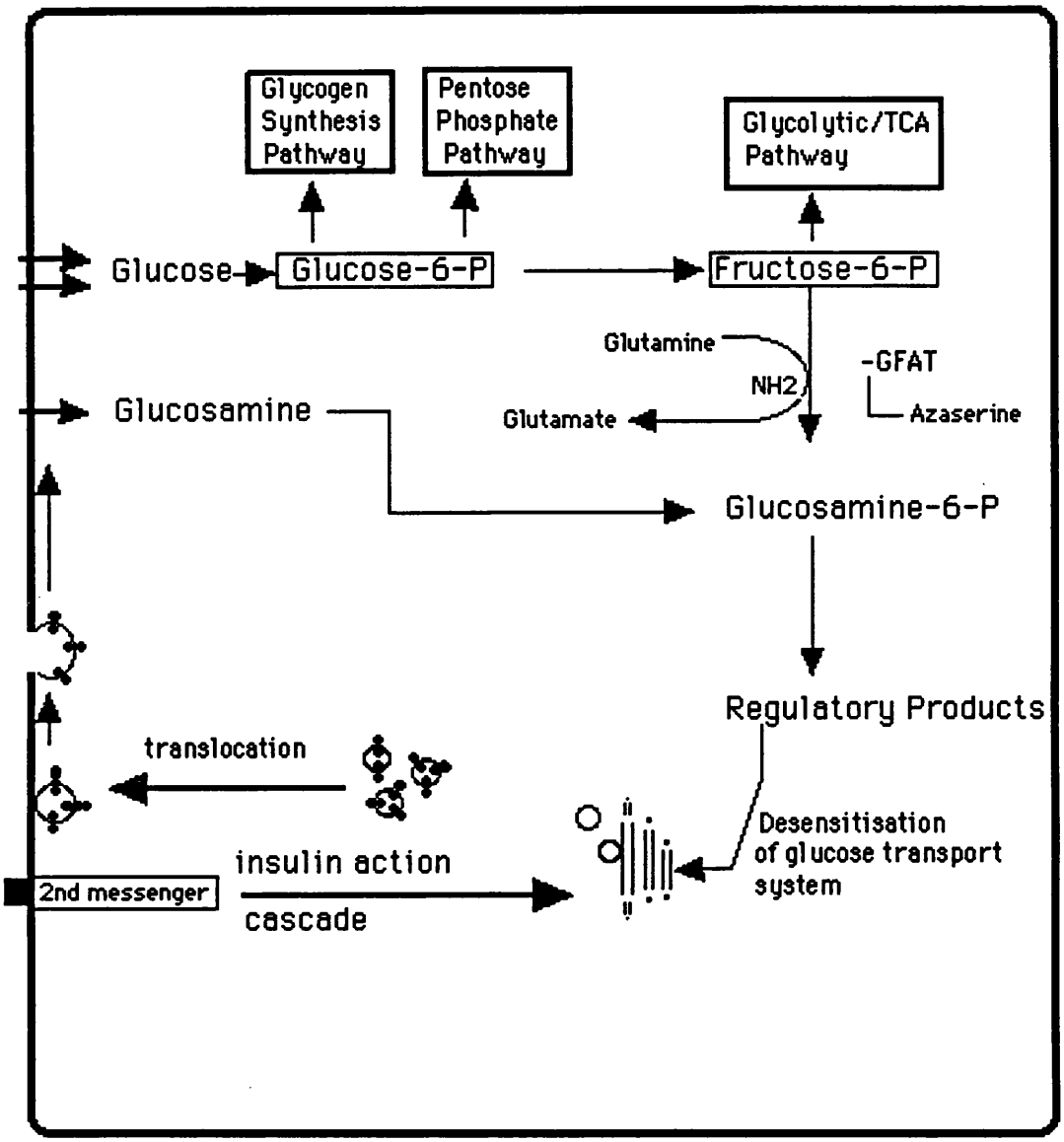
O-diazoacetyl-L-serine concentration (μM)	Basal	30 min Insulin	Fold increase
0	12 ± 2.5	301 ± 12	25
30	10 ± 3	299 ± 21	29.9
75	12 ± 3	305 ± 9	25.4
150	12 ± 2.5	310 ± 12	25.8

(d)

6-Diazo-5-oxonorleucine concentration (μM)	Basal	30 min Insulin	Fold increase
0	12 ± 2.5	301 ±12	25
30	11 ± 1.9	294 ± 9	26.7
75	11 ± 2	289 ± 13.5	26.2
150	13 ± 4	307 ± 7.5	23.6

FIGURE 5.6

PROPOSED MODEL MEDIATING GLUCOSE-INDUCED DESENSITISATION OF GLUCOSE TRANSPORT



This is the model proposed by Marshall *et al.* depicting the metabolic pathway mediating glucose-induced desensitisation of the glucose transport system and the postulated sites of azaserine and glucosamine action.

5.4 DISCUSSION

In previous studies carried out by Gould *et al.* it was demonstrated that the human HepG2 transporter, which is not insulin-sensitive in its native cell type, exhibited insulin-stimulated translocation when expressed in 3T3-L1 adipocytes. Moreover, it was demonstrated that both the HepG2 transporter and native GLUT 1 transporter translocated, from an intracellular region to the plasma membrane, to a similar extent. It was suggested from these data that the insulin-induced translocation of glucose transporters in adipocytes was not dependent on the transporter species but that specific factors unique to the adipocyte cell regulated the translocation process (Gould *et al.*, 1989). Hence from this assumption it could be postulated that GLUT 2, or indeed any other transporter species, could be rendered insulin-responsive when expressed in 3T3-L1 adipocytes.

This study was undertaken in an attempt to determine the subcellular localisation and insulin-responsiveness of GLUT 2, the liver-type transporter, when expressed in a fat cell environment. By preparing subcellular membrane fractions from GLUT 2-transfected 3T3-L1 adipocytes it was demonstrated that GLUT 2 was predominantly plasma membrane-localised and that only low levels were detectable in low density microsomal fractions. Thus the protein appeared to be targeted to the plasma membrane as it would be in its native cell.

Other groups have reported that glucose transporter targeting in transfected cells is similar to that observed in the transporters' native cell. Results from studies carried out in HepG2, CHO and 3T3-L1 fibroblast cells transfected with GLUT 1 and GLUT 4 cDNAs have shown that GLUT 1 and GLUT 4 targeting was to plasma membrane and intracellular regions respectively (Shibasaki *et al.* ,1992; Hudson *et al.*, 1992; Robinson *et al.*, 1992) (see chapter 4 for a more detailed discussion). However in contrast to the study presented here, these transfection studies were carried out using fibroblastic cell lines, none of which were representative models of a highly insulin-sensitive environment.

Taken together, the data from this study and from the studies mentioned above (exclusive of the study by Gould *et al.*,1989) suggest that targeting information may be contained in the primary sequence of glucose transporters and that cell environment does not dictate subcellular localisation.

Results from 2-deoxyglucose uptake assays showed that, compared to 3T3-L1 adipocytes, basal 2-deoxyglucose uptake values were 3.9 and 2.5 -fold higher in 3T3/G2a and 3T3/G2b adipocytes respectively (Table 5.3). These results were consistent with the data showing that GLUT 2 protein levels in 3T3/G2a cells were higher relative to levels in 3T3/G2b cells (Table 5.1).

The insulin-stimulated rates of 2-deoxyglucose uptake observed in GLUT 2-expressing adipocytes were lower however, relative to the insulin-stimulated uptake rates observed in wild-type cells. Upon insulin stimulation, 2-deoxyglucose uptake rates in 3T3/G2a and 3T3/G2b adipocytes were 132 ± 18 and 140 ± 32 pmol/million cells/min respectively. These were in contrast to the insulin stimulated 2-deoxyglucose uptake rates measured in wild-type cells which were 293 ± 16 pmols/million cells/min. Therefore it appeared that GLUT 2-expressing adipocytes demonstrated a reduced sensitivity to insulin with respect to 2-deoxyglucose uptake (see discussion).

Furthermore, it should be noted that the mean -fold increase in 2-deoxyglucose uptake in response to insulin was considerably lower in GLUT 2 expressing clones relative to wild-type cells. This was because the basal rates of 2-deoxyglucose uptake were higher, and the insulin-stimulated 2-deoxyglucose uptake rates were lower in GLUT 2-expressing clones relative to wild-type cells (see Table 5.4a). Upon exposure to insulin, uptake was only increased 3 and 9 -fold over basal uptake in 3T3/G2a and 3T3/G2b cells respectively, which was in contrast to the 20 to 30 -fold increase in uptake observed in wild-type cells.

Immunoblotting of subcellular fractions prepared from insulin-stimulated 3T3/G2a, 3T3/G2b and wild-type cells, with anti-GLUT 4 antibodies revealed that the increase in the percentage of GLUT 4 at the plasma membrane was similar for all

cells. Hence the extent of translocation of GLUT 4 from the LDM to the PM appeared to be similar in both wild-type and GLUT 2-transfected cells (see Tables 5.2a, b and c).

However from these immunoblotting studies alone it was not possible to determine if the absolute amount of GLUT 4 expressed per μg of total protein was identical in both GLUT 2-expressing and non-transfected cells. Therefore, total cell membranes were prepared from basal wild-type cells, clone 3T3/G2a and clone 3T3/G2b, and the amount of GLUT 4/μg of total protein was calculated for each. It was possible that the significantly lower rate of insulin-stimulated 2-deoxyglucose uptake in 3T3/G2a may have resulted from a reduction in the absolute amount of GLUT 4 protein expressed. Results however, indicated that GLUT 4 levels were 0.6-0.8ng GLUT 4/μg total protein in both 3T3/G2a and wild-type adipocytes and that there was no apparent reduction in GLUT 4 expression in 3T3/G2a adipocytes (fig 5.5). Thus the expression of GLUT 2, a high capacity transport protein with respect to glucose uptake, did not appear to induce any significant down-regulation of the native insulin-regulatable glucose transporter GLUT 4 as would maybe have been expected.

The lower rates of 2-deoxyglucose uptake observed in GLUT 2-expressing cells may have been the result of glucose-induced desensitisation of the glucose transport system, a phenonomen first described by Marshall *et al.* (1991). These investigators proposed that a unique metabolic pathway exists in adipocytes, the products of which lead to "insulin-resistance" (reduced sensitivity to insulin) by impairing insulin-induced glucose transporter translocation. This metabolic pathway, termed the hexosamine pathway, is shown schematically in Fig. 5.6. Results from the studies by Marshall *et al.* (1991) showed that in addition to glucose and insulin, glutamine was required to induce desensitisation of the insulin-responsive glucose transport system. The key role of glutamine appeared to be as a substrate for the first and rate-limiting enzyme of the hexosamine pathway, glutamine:fructose 6-phosphate amidotransferase (GFAT), which uses the amide group of the amino acid to convert

fructose-6-phosphate to glucosamine 6-phosphate. Glucosamine itself was also found to be a very potent inducer of "insulin resistance" independent of the presence of glutamine.

Marshall *et al.* (1991) postulated that as the glucose concentration exceeds the capacity of the major glucose utilising pathways such as glycolysis and the pentose-phosphate pathway, fructose 6-phosphate levels would rise and more glucose would be forced to flow through alternative pathways such as the hexosamine pathway. This enhanced flux through the hexosamine pathway would, through the production of some unknown inhibitory factor(s), then feedback inhibit insulin-stimulated glucose uptake. It could be envisaged that the expression of GLUT 2 in adipocyte clones 3T3/G2a and 3T3/G2b promoted desensitisation of the glucose transport system through activation of the hexosamine pathway. GLUT 2 expression at the plasma membrane in transfected adipocytes resulted in an increased basal flux of glucose relative to that observed in wild-type adipocytes. As a result, glucose influx may have exceeded the capacity of the major glucose utilising pathways in GLUT 2 -transfected adipocytes and thus some of the incoming glucose may have been routed to the hexosamine pathway. The presence of glutamine, amongst many other amino acids in DMEM medium bathing the cells, would have ensured that there was a steady supply of glutamine for the pathway to be utilised.

To investigate the possibility that the "insulin-resistance" or reduced sensitivity to insulin observed in GLUT 2-expressing adipocytes was a direct effect of glucose metabolism through the hexosamine pathway, an attempt was made to inhibit the pathway and to then determine if insulin sensitivity had been restored to normal. Cells were incubated overnight in growth medium containing various concentrations of O-diazoacetyl-L-serine (azaserine) or 6-diazo-5-oxonorleucine, both inhibitors of glutamine:fructose 6-phosphate amidotransferase (GFAT) and the following day basal and insulin-stimulated 2-deoxyglucose uptake rates were measured in clones 3T3/G2a and in wild-type cells (see Tables 5.5a, b, c and d).

Results showed that in 3T3/G2a, 3T3/G2b and wild-type adipocytes both basal and insulin-stimulated 2-deoxyglucose uptake measurements were unchanged relative to corresponding measurements observed in untreated cells.(see Tables 5.5a, b, c and d). Hence results suggested that the reduced sensitivity to insulin observed in clones 3T3/G2a and 3T3/G2b had not been reversed and therefore could not be attributed to glucose metabolism through the hexosamine pathway.

However, as mentioned previously, Marshall *et al.* (1991) demonstrated that glucosamine was also found to be a potent inducer of insulin-resistance and in addition its potency was 40 times greater than that of glucose. It was demonstrated that, in adipocytes, glucosamine could induce desensitisation in the absence of glutamine and in the presence of GFAT inhibitors. Therefore it was concluded that glucosamine entered the hexosamine-desensitisation pathway at a point distal to GFAT amidation (see Fig. 5.6).

On the basis of these findings, it could be postulated that GLUT 2 expression in transfected 3T3-L1 adipocytes had promoted a glucose-induced reduction in insulin sensitivity through the hexosamine pathway by mediating the influx of glucosamine directly. Due to the limitations of time however, this hypothesis could not be investigated further, but this work will be completed at a future date by Gould and co-workers. These investigations will utilise the *Xenopus* oocyte expression system to determine the potential role of GLUT 2 in mediating the uptake of glucosamine. GLUT 2 mRNA will be microinjected into *Xenopus* oocytes and glucosamine uptake will be measured by a competition assay where the uptake of [^{3}H] 2-deoxyglucose will be assessed in the presence of increasing concentrations of glucosamine. If results from these studies indicate that the V_{max} of GLUT 2 for [^{3}H] 2-deoxyglucose uptake is reduced in the presence of glucosamine then this will indicate that the amino acid is a substrate of the GLUT 2 transporter. As a control experiment to assess any spurious results arising from glucose transporter over-expression on glucosamine uptake, GLUT 1 mRNA will also be microinjected into oocytes and glucosamine uptake will be determined as described previously. In

addition glutamine uptake will be assessed by the same method to determine if GLUT 2 is able to mediate the transport of this amino acid substrate.

It could be postulated that GLUT 2 would be able to function as a transporter of glucosamine since the structure of this amino sugar is comparable with that of 2-deoxyglucose, with both structures having a substitution at the C-2 position. However it may be the case that the presence of an amino group at position C-2 would increase the positive charge of this substrate thus interfering with its transport via GLUT 2. Having encountered the reduced sensitivity to insulin in GLUT 2-transfected adipocytes it would be interesting to determine if this sensitivity could be restored to "normal" by introducing into these cells the cDNA encoding glucokinase, the high K_m hexokinase which is normally expressed in cells where the GLUT 2 glucose transporter isoform is present.

The hexokinases (ATP:hexose 6-phosphotransferases) constitute a family of structurally related enzymes which are expressed in a highly regulated tissue-specific manner. To date, 4 hexokinases have been identified and hexokinases I, II and III share several properties including a high affinity for glucose and inhibition by the reaction product glucose 6-phosphate (Katzen and Schimke, 1965; Grossbard and Schimke, 1966).

Hexokinase IV, also known as glucokinase, differs from the other members of the family in that it is not inhibited by glucose 6-phosphate and it exhibits a lower affinity for glucose (Weinhouse, 1976). Glucokinase is expressed only in the liver and the β–cells of the pancreas (Magnuson and Shelton, 1989; Iynedjian *et al.*, 1989) and phosphorylates glucose which crosses the cell membrane by GLUT 2-mediated transport.

Hexokinase II is the isoform expressed in insulin-sensitive tissues such as skeletal and cardiac muscle and adipose tissue (Katzen 1967; Katzen *et al.*, 1968; Sochor *et al.*, 1990) Glucokinase has a lower affinity for glucose (6mM) and thus a greater capacity for glucose than its counter-part HK II which exhibits a K_m of 150μM (Iynedjian, 1993). Thus, it could be expected that, in adipocytes, if glucose

flux was increased to beyond a concentration which could be phosphorylated by HK II then some of the glucose may indeed be routed to alternative pathways such as the hexosamine pathway. However it could be postulated that if glucokinase was expressed in adipocytes in addition to HK II, then the amount of glucose which could be phosphorylated would increase and as a consequence, the capacity of the normal glucose utilising pathways would be increased. Therefore no glucose would have to be routed to alternative pathways.

It would be interesting in the future, carrying on from this study, to express glucokinase in GLUT2-transfected adipocytes to determine if the reduced sensitivity to insulin observed in these cells could be reversed. It could be envisaged that a hexokinase with a higher K_m would work more efficiently in cells where glucose flux was increased and that consequently, the phosphorylation of glucose would not be rate-limiting. Thus in GLUT 2-transfected cells expressing glucokinase, glucose would no longer need to be routed to alternative pathways.

The overall conclusion however, on the basis of the data presented here, is that the expression of GLUT 2 in 3T3-L1 adipocytes promotes a reduction in insulin-stimulated glucose transport which cannot be directly attributed to down-regulation in GLUT 4 protein expression. Hence the nature of this reduced "insulin-sensitivity" remains to be elucidated.

CHAPTER 6

THE EXPRESSION OF GLUT 4 IN H4IIe CELLS TO STUDY GLUT 4 INSULIN-RESPONSIVENESS

6.1 INTRODUCTION

The H4IIe cell line was derived from the rat H35 Reuber hepatoma cell line by Morse in 1962 and since that time has been used extensively as model for hepatocytes in culture (Pitot *et al.*, 1964). These cells present a number of advantages over primary cultured hepatocytes in that they exhibit a high growth rate, experimental durability and are easily maintained in culture.

When viewed under the light microscope H4IIe cells are similar in appearance to epithelial cells. When viewed in more detail under scanning electron microscopy the nuclei of H4IIe cells appear larger than those of normal hepatocytes. This probably reflects the ability of H4IIe cells to grow rapidly in culture (Hofmann *et al.*, 1980).

H4IIe cells have approximately 10,000-30,000 insulin receptors per cell which is less than the number present in normal hepatocyte cells (Hofmann *et al.*, 1980). H4IIe cells are nonetheless highly responsive to insulin and studies have demonstrated that maximal stimulation of D-[U-^{14}C]glucose incorporation into glycogen occurs when cells are incubated in the presence of 10nM insulin. Furthermore, this maximal insulin response by H4IIe cells results in a 2 to 3 -fold increase in glucose incorporation into glycogen over levels measured under basal conditions (Hofmann *et al.*, 1980).

Although the characteristics and cellular responses are not entirely similar to those of normal hepatocytes, hepatoma cell lines such as the H4IIe, HepG2, FAO and H35 cell line have been used extensively in the study of insulin action and have proved useful as hepatocyte model systems. For example, HepG2 cells have been

widely used in the study of insulin transmembrane signalling (Hatada *et al.*, 1989) and in the regulation of gene transcription.

The liver is an important site of glucose uptake and storage, and mobilisation of glucose from this tissue helps maintain glucose homeostasis (Sacca, 1987). Early studies using isolated hepatocytes indicated that glucose uptake by these cells occurred by a low affinity, high capacity facilitative glucose transport system that was not acutely modulated by insulin (Ciaraldi *et al.*, 1986). The liver protein responsible for this activity was subsequently identified, cloned, characterised and designated GLUT 2 (Thorens *et al.*, 1988; Fukomoto et al., 1988).

Studies utilising immunocytochemical techniques have since shown that GLUT 2 is expressed in all hepatocytes, where its expression is restricted to the sinusoidal plasma membrane (Thorens *et al.*, 1990). As mentioned in previous sections (see 1.2.3 and 4.1) GLUT 2 has the lowest affinity and highest transport capacity for glucose of all the transporter isoforms and mediates the bi-directional movement of glucose into and out of the hepatocyte. Following a carbohydrate meal blood glucose levels rise and, as a consequence, glucose is transported into the liver where it is converted to glycogen for energy storage.

When blood glucose levels fall, the glycogen energy reserve is broken down and the resulting glucose is transported out of the liver into the blood via the GLUT 2 transporter isoform.

GLUT 1, the transporter expressed in most tissue types, is also present at high levels in transformed cell lines. It is normally undetectable or expressed at very low levels in liver but its mRNA has been detected in liver from fasted (Rhoads *et al.*, 1988) and foetal rats (Werner *et al.*, 1989). Rhoads *et al.* also demonstrated that hepatocytes, when placed in culture, were capable of inducing synthesis of GLUT 1 mRNA. It was demonstrated that GLUT 1 mRNA levels were increased in hepatocytes after their removal from liver and plating in culture (Rhoads *et al.*, 1988). Thorens *et al.* showed that GLUT 1 transporter was detectable in hepatocyte cells in the livers of normal rats although at low levels (Thorens *et al.*, 1990).

H4IIe cells as mentioned previously have been widely used as a model system to study various aspects of insulin action. However, these cells are not identical in all respects to normal hepatocytes. The most striking difference between H4IIe cells and hepatocytes is their pattern of glucose transporter expression. As mentioned here, and in preceding sections, hepatocyte cells express the GLUT 2 transporter isoform at high levels. Studies by Gould *et al.* have shown that, in contrast, this isoform was undetectable in membranes prepared from H4IIe cells, however GLUT 1 protein was detectable at high levels (Gould *et al.*, unpublished data). Thus, in culture H4IIe cells express only the GLUT 1 transporter isoform unlike hepatocytes which express GLUT 2. However H4IIe cells, like hepatocytes, do not exhibit increased glucose uptake in response to insulin.

This study was carried out to determine the insulin responsiveness and contribution to cellular glucose uptake of GLUT 4, the insulin-sensitive glucose transporter, when expressed in a liver-type cellular environment. In normal hepatocytes GLUT 2 is expressed at the plasma membrane of the cell and glucose transport is insensitive to stimulation with insulin. Thus, by inference, GLUT 2 does not exhibit insulin-induced translocation.

Hence this part of the investigation was the complement of the study carried out previously where 3T3-L1 cells were transfected with the liver cell transporter GLUT 2.

The results from these studies together, expressing GLUT 2 in an adipocyte environment and GLUT 4 in a liver cell environment, should provide information regarding the influence of cell environment and/or glucose transporter protein sequence in dictating glucose transporter insulin-responsiveness and translocation.

Previous studies carried out on GLUT 4-transfected cells have demonstrated that GLUT 4 protein targeting was similar to that in its native cell in that it was directed to an intracellular compartment (see 4.1). Investigators, however, reported no demonstration of GLUT 4 translocation to the cell surface with a concomitant increase in glucose uptake when transfected cells were stimulated with insulin. The

results of these studies therefore suggested that factors present within the adipocyte environment were both necessary and important in the process of insulin-induced translocation. On the basis of these results it could therefore be postulated that GLUT 4 in a liver cell environment would not undergo translocation since factors present in the adipocyte environment would most probably be absent from a liver cell.

Consequently if the results of this study indicate that, in a liver cell environment, GLUT 4 is able to undergo insulin-stimulated translocation concomitant with an increase in glucose transport, this could represent the first demonstration of reconstituted, insulin-stimulated glucose transport.

6.2 METHODS

6.2.1 Construction of the expression vector containing GLUT 4 cDNA

pGEM.AMT, containing the cDNA encoding the full length human GLUT 4 protein was a gift from Dr. Graeme Bell, University of Chicago.

GLUT 4 cDNA was isolated from pGEM.AMT by restriction digestion. The vector was digested for 60 min at 37°C with *Sal* I in a reaction volume of 20µl. After this time, 5µl of 5X DNA loading buffer was added to the digestion mix and the entire volume was electrophoresed on a 1% agarose gel. Included also on the gel was a sample of *Bst E* II- digested λ ladder so that the size of DNA fragments generated from digested pGEM.AMT could be determined. After electrophoresis, the gel was viewed under U.V. illumination and the sizes of the DNA fragments were determined. Two bands were present in the digested pGEM.AMT sample, the larger of the two at 3Kbp representing the parent vector, pSPT64 and the smaller band at 2100bp representing the fragment of DNA encoding GLUT 4.

The band corresponding to GLUT 4 DNA was excised from the gel using a scalpel blade and the gel chip was subjected to the "Gene Clean" procedure in order to isolate and purify the isolated DNA. Following this procedure, 1µl of the isolated DNA was mixed with 5µl of 5X DNA loading buffer and the sample was electrophoresed on a 1% agarose gel to verify that the yield of DNA was sufficient to perform subsequent subcloning steps.

6.2.2 Preparation of the expression vector LK444 for ligation to GLUT 4 cDNA

5µg LK444 was digested with 1 unit of *Sal* I at 37°C for 60 min as previously described (see 2.7.6), and treated with 1µg of RNase for a further 30 min at 37°C. Following phenol extraction and ethanol precipitation (see 2.7.1), LK444

DNA was treated with calf alkaline phosphatase to inhibit self-ligation of the vector (see 2.7.7). 1µl of CIP-treated DNA was then electrophoresed on a 1% agarose gel to verify that the DNA had been linearised.

6.2.3 Ligation of phosphatase-treated LK444 DNA and GLUT 4 cDNA

GLUT 4 was ligated with linearised LK444 at a molar ratio of 10:1 using the method previously described (see 2.7.8).

Following this, 200µl of competent *E. coli* cells were transformed with 1µl of the ligation mix, the cells spread at several dilutions on 2xYT plates, containing ampicillin at 25µg/ml, and the plates then incubated overnight at 37°C. In addition to the ligation transformation reaction, control transformation reactions were also assembled where competent *E.coli* cells were transformed with either 100ng linearised LK444 DNA or with 10µl H_2O (see 4.2.3). The following morning a number of ampicillin-resistant colonies were individually selected from the ligation mix 2xYT/ampicillin plate and each was used to inoculate 2ml of 2xYT medium containing 25µg/ml ampicillin. These mini-preps were grown overnight at 37°C and the following morning plasmid DNA isolated from the *E. coli* by alkaline lysis. The plasmid DNA was then purified by phenol extraction and ethanol precipitation (see 2.7.1). 1µl of the isolated DNA was then electrophoresed on a 1% agarose gel together with a sample of *Bst E* II-digested λ ladder to check the efficiency of DNA recovery and verify the size of the construct.

6.2.4 Restriction digestion of LK444/GLUT 4 to determine GLUT 4 cDNA orientation

Restriction digestion reactions were performed on ligated LK444/GLUT 4 cDNA samples to determine if the GLUT 4 insert was present and, if so, to determine its orientation within the expression vector. Restriction digestions were assembled

TABLE 6.1

RESTRICTION DIGESTION TABLE FOR LK444/GLUT4

To determine the orientation of GLUT 4 cDNA within LK444/GLUT 4, restriction digestion reactions were assembled as outlined below. The sizes of the DNA fragments expected from LK444/GLUT 4 digestions, when GLUT 4 is ligated in the correct or incorrect orientation are shown

Restriction enzyme	**fragment size (bp) (correct orientation)**	**fragment size (bp) (incorrect orientation)**
Eco R1	5885, 551, 5700	2300, 7836
Sal 1	10,000, 2136	10,000, 2136
Bgl II/ *Bam* H1	11,585, *551	10,551, 1585

*Definitive digest fragment: If GLUT 4 is in the correct orientation then this small fragment should be present.

using the combination of enzymes specified in Table 6.1 and the sizes of the DNA fragments generated from the reactions were determined.

Table 6.1 shows the sizes of the fragments expected from each digestion reaction when GLUT 4 is ligated to LK444 DNA in the correct (sense) and incorrect (antisense) orientation.

6.2.5 Large scale preparation of LK444/GLUT 4

Once LK444/GLUT 4 DNA containing GLUT 4 in the correct orientation had been identified, the mini-prep from which it was first isolated was used to inoculate 500ml 2xYT medium for a large scale preparation of the plasmid (see 2.7.4). Following this, the concentration and purity of the isolated DNA was determined and restriction digestions were performed, as before, to verify that the correct construct had been isolated (see 2.7.5). The DNA was then sequenced using the Applied Biosystems sequencing system (see 4.2.6).

6.2.6 Determination of G418 concentration for selection of transfected H4IIe cells

In order to determine a suitable concentration of G418 in which to maintain H4IIe cells after transfection, wild-type H4IIe cells were grown in 10% foetal calf serum/DMEM containing 0, 0.25, 0.5, 0.8, 1.0, or 1.2mg/ml G418. Cells were monitored each day and the number of surviving cells was noted.

The time course for cell death was very similar to that observed for 3T3-L1 fibroblasts (see 4.2.7). Within 7 days all cells grown in medium containing 0.8-1.2mg/ml G418 had perished while cells grown in lower concentrations appeared healthy. However after 10-14 days the number of surviving cells grown in 0.25 and 0.5mg/ml G418 had decreased in number while cells grown in the absence of G418 continued to appear healthy.

From this data it was decided that initially cells would be selected in 0.5mg/ml Geneticin since at this concentration the effect was lethal but not instantaneous. Following the appearance of discrete antibiotic-resistant colonies it was decided that the G418 concentration would be reduced to 0.25mg/ml (see 4.2.7).

6.2.7 Transfection of H4IIe cells with LK444/GLUT4 DNA

H4IIe cells were grown to 70% confluency in 10cm cell culture dishes containing 10% foetal calf serum/DMEM medium, and then transfected with 5μg of LK444/GLUT 4 DNA/plate using the DOTAP lipofection procedure (see 2.8.10). Two days post-transfection, 5ml of the growth medium was removed and replaced with medium containing G418 such that the final concentration of G418 per plate was 0.5mg/ml. This procedure was repeated every 2 days.

After 7 days most of the cells had perished, although a number of single surviving cells were present on the plate. At this stage the G418 concentration was reduced to 0.25mg/ml to allow expansion of the surviving antibiotic-resistant colonies.

Following another 5-7 days of careful feeding, individual colonies were gently trypsinised and replated on 6cm plates containing G418-free growth medium. The next day, 2ml of the growth medium was replaced with medium containing G418 such that the final concentration of antibiotic per plate was 0.25mg/ml.

The cells were then grown to 70-80% confluency after which time they were trypsinised and used to seed three to five 10cm plates. These cells were grown to confluency in 10% foetal calf serum/ DMEM medium containing G418 at 0.25mg/ml, trypsinised, frozen down and then stored at -80°C as cell stocks (see 2.8.5). In addition a cell lysate was prepared from one plate of each clone so that the sample could be screened for the expression of GLUT 4.

6.2.8 Screening of GLUT 4-transfected H4IIe cells for the expression of GLUT 4 protein

A number of cell lysates, prepared from individual clones, were subjected to electrophoresis on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. The membranes were then incubated in 5% non-fat milk/1st wash buffer (to block non-specific sites on the nitrocellulose) and then incubated with anti-GLUT 4 antiserum at 10μg/ml in 1% non-fat milk/1st wash buffer (see 2.5.1). Nitrocellulose membranes were then developed using the ECL detection system to facilitate the detection of low levels of expressed protein (2.6.2).

6.2.9 Measurement of [^{3}H] 2-deoxyglucose uptake in transfected and non-transfected cells

Non-transfected and GLUT 4 transfected H4IIe cells were grown to confluency on 6-well plates containing in 10% foetal calf serum/ DMEM medium with G418 at a concentration of 0.25mg/ml. 2h prior to assay, the cells were incubated in serum-free medium after which time the uptake of 0.1mM [^{3}H] 2-deoxyglucose was measured. Uptake was assessed in the absence or presence, for 30 min, of 1μM insulin. Non-specific [^{3}H] 2-deoxyglucose uptake was corrected for by subtracting uptake measured in the presence of 10μM cytochalasin B, a potent inhibitor of glucose uptake (see 2.10).

6.2.10 Quantitation of GLUT 4 protein expressed per mg of total protein in GLUT 4-expressing clone 5A cells

On the same day that H4IIe clone 5A cells, plated on 6-well plates, were assayed for [^{3}H] 2-deoxyglucose uptake, cell lysate samples and samples for protein determination were prepared from 3 wells of a duplicate 6-well plate.

Protein concentrations were determined according to the method of Peterson (1977) (see 2.2.1).

Prepared cell lysates and GLUT 4 protein standards were subjected to electrophoresis on 10% SDS/polyacrylamide gels and proteins were transferred to nitrocellulose membranes. The membranes were incubated in 5% non-fat milk/1st wash buffer (to block non-specific binding sites on the nitrocellulose) and then probed with 10μg/ml affinity-purified anti-GLUT 4 antibodies in 1% non-fat milk/1st wash buffer. Membranes were then developed using the ECL detection system.

To quantify ECL immunoblot signals from lysate samples and from GLUT 4 standards, immunoblot autoradiographs were subjected to scanning densitometry. The amount of GLUT 4 expressed in lysate samples was determined relative to the known amount in GLUT 4 standards and results were expressed as ng GLUT 4 expressed/μg total protein.

6.2.11 [^{3}H] ATB-BMPA labelling of GLUT 4 in GLUT 4-transfected H4IIe clone 5A cells

GLUT 4-transfected H4IIe cells, grown to confluency on a 6-well plate, were incubated in serum-free DMEM medium for 2 hours at 37°C and then for a further 60 min in the presence or absence of 1μM insulin. After terminating insulin stimulation by aspirating the incubation medium and washing the cell monolayers 3 times with 3ml of ice-cold PBS pH 7.4, the plates were incubated on ice in preparation for labelling.

[^{3}H]-2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis-(D-mannose-4-yloxy)-2-propylamine ([^{3}H] ATB-BMPA) label used in the investigation described here was a gift from Dr G. D. Holman, University of Bath.

In a dark room, 300μl of [^{3}H] ATB-BMPA label (5mCi/ml) was diluted 1:1(v/v) with PBS pH 7.4, 100μl of the mixture was added to each well and the plate was wrapped in aluminium foil. Photolysis was then carried out in a RPR 200

Rayonet reactor at maximum power (1.6mW/cm^2) for 60 seconds (2 x 30 second bursts). The cells were washed 3 times with 1ml of PBS pH 7.4, and then solubilised in 1.4ml/well of 1% (w/v) Thesit/PBS pH 7.4 containing 1μg pepstatin A, 0.2mM DFP and 4μM Ep 64 protease inhibitors. After incubation on ice for 5 min, the solubilised cells were transferred to 1.5 ml microfuge tubes and the nuclei were pelleted by microfuging at maximum speed for 5 min. The supernatants were then retained and transferred to fresh microfuge tubes.

6.2.12 Immunoprecipitation of [^{3}H] ATB-BMPA-labelled GLUT4 protein in H4IIe clone 5A cells

Anti-GLUT 4 antiserum (10μl) was added to each tube of solubilised cells, the samples vortexed and incubated on ice for 90 min. 40μl of Protein A agarose, which had been pre-washed with 0.5ml 1% Thesit /PBS pH 7.4 was then added and samples were rotated at slow speed at 4^{o}C for 2h.

After microfuging at maximum speed for 10 seconds, the protein A agarose pellet was retained, 1ml of 1% Thesit/PBS pH 7.4 was added and the samples vortexed then microfuged for 10 seconds. The supernatant was discarded and the pellet washed once with 1ml of 1%(w/v) Thesit/PBS pH 7.4. and once with 1ml of 0.1% (v/w) Thesit/PBS, pH 7.4. 100μl of 1.3X sample buffer (1.67ml 4X sample buffer, 2.4g urea (8M final concentration), 1.5ml H_2O, 20mM DTT) was added to the pellet and the samples were incubated at room temperature for 20 min. (1.3X sample buffer was added since H_20, contained in the swollen agarose beads, would be released during incubation which would dilute the sample buffer to a final concentration of 1X). Finally, after microfuging at maximum speed for 10 seconds, the supernatant was retained, snap frozen and stored at -80^{o}C until required.

6.2.13 Quantitation of plasma membrane GLUT 4 protein in basal and insulin-stimulated cells by measuring GLUT 4 [^{3}H] ATB-BMPA label incorporation

[^{3}H] ATB-BMPA-labelled immunoprecipitated samples were electrophoresed on large 12.5% SDS/polyacrylamide gels and gel lanes sliced into 0.5cm slices. The position of each slice, relative to the positions of the molecular weight markers, was noted and the gel chips placed in scintillation vials.

The gel chips were dried in an oven at 50°C for 2-3 hours. 1:17 (v/v) 880 Ammonia/hydrogen peroxide (0.5ml) was added to each sample, and the vials were heated again in a 50°C oven until the gel chips were completely solubilised. Finally 10ml of scintillation fluid was added to each sample and tritium incorporation was determined by liquid scintillation counting.

6.2.14 Trypsinisation of cell surface GLUT 4 in transfected H4IIe cells to demonstrate insulin-stimulated GLUT 4 translocation

Confluent H4IIe cells, grown on 6-well plates, were incubated in serum-free medium for 3 hours at 37°C. Following this, the cells were incubated in the absence or presence, for 30 min, of 1μM insulin. Cells were then treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Chem. Co) for 15, 30 or 60 min. After the appropriate treatment times, the trypsin digestion reactions were quenched with the addition of 4mg/ml soybean trypsin inhibitor (Sigma Chem Co) in KRP, pH 7.4. Following this, the cells, which had lifted from the culture plate wells during trypsin treatment, were titurated gently in 4ml of 2mg/ml soybean trypsin inhibitor/KRP and transferred to 5ml polyethylene tubes. The cells were then pelleted at 1000 rpm for 10 min and pellets resuspended in 2ml of 2mg/ml (w/v) soybean trypsin inhibitor/KRP.

This washing procedure, with centrifugation between washes, was repeated a further two times. Finally, following the final centrifugation step, the inhibitor wash was aspirated and each cell sample resuspended in 600µl of lX SDS Laemmli sample buffer.

Samples were then subjected to electrophoresis on 12.5% polyacrylamide gels and proteins transferred to nitrocellulose (see 2.3 and 2.4). The membranes were then probed with anti-GLUT 4 antibodies and the blots developed using the ECL detection system. (see 2.5.1 and 2.6.2). The relative amount of GLUT 4 was quantified by densitometric scanning of the immunoblot signals obtained from ECL-developed autoradiographs. The immunoblot signals from the lower molecular weight species, representing trypsinised GLUT 4 protein, were quantitated relative to signals from intact undigested GLUT 4.

6.3 RESULTS

6.3.1 Restriction digestion of LK444/GLUT 4 DNA

Restriction digestion of LK444 DNA with *Sal* I enzyme alone revealed that, as expected, only 1 site for the enzyme, located in the unique cloning region, was present in the plasmid. Similarly, digestion with either *Eco* RI or *Bam* HI yielded only one DNA fragment indicating that only one site for each enzyme was present in the vector. Digestion with *Bgl* II however revealed that no sites for this enzyme were present within the plasmid (data not shown).

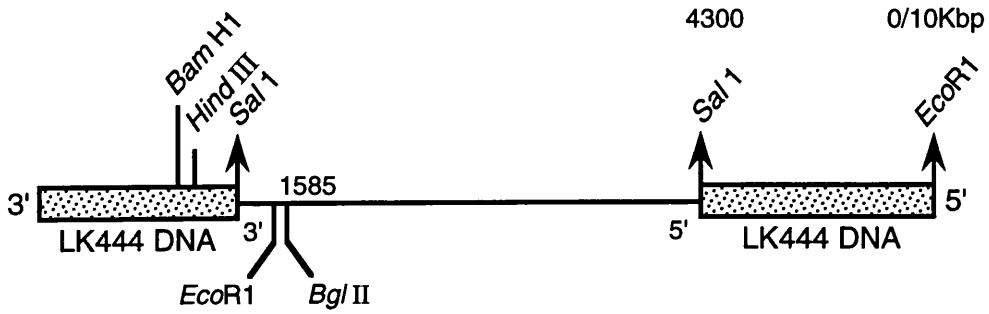
Restriction digestion reactions, using the combination of enzymes specified in Table 6.1, were carried out on two LK444/GLUT 4 DNA samples designated 14 and 8, isolated from 2 selected transformed *E.coli* colonies (2.7.6). Results shown in Fig. 6.2a indicated that both samples contained GLUT 4 insert but that sample 14 contained the DNA in the correct orientation while sample 8 contained the insert in the reverse orientation. A fragment of approximately 500bp in size (from co-ordinate 1585 in the insert to co-ordinate 4300 in the plasmid), (Fig. 6.1) was obtained in sample 14 upon digestion with *Bgl* II/*Bam* HI which was confirmation that the insert was present in the correct orientation in the plasmid (Fig. 6.2b). This small fragment however, was only detectable after intense and prolonged staining with ethidium bromide. No fragment of 500bp in size was obtained in sample 8 upon *Bgl* II/*Bam* HI digestion even after the agarose gel was incubated overnight in ethidium bromide (data not shown). This suggested therefore that the plasmid isolated from colony 8 contained GLUT 4 but in the incorrect orientation (Fig. 6.2b and Fig. 6.1).

Sample 14 DNA was then sequenced using the Applied Biosystems automated sequencer system by the procedure outlined by the manufacturers (see section 4.2.6). Results confirmed that the construct contained full length GLUT 4 cDNA in the correct orientation (data not shown).

Figure 6.1

RESTRICTION MAP OF LK444 / GLUT 4

LK444 WITH GLUT 4 cDNA IN THE CORRECT ORIENTATION



LK444 WITH GLUT 4 cDNA IN THE INCORRECT ORIENTATION

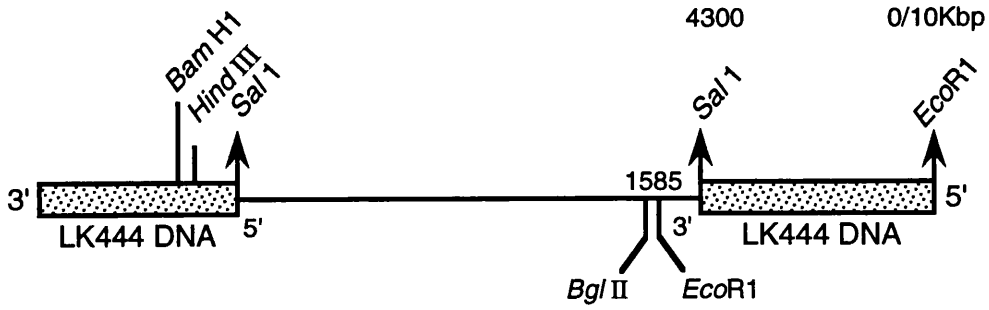
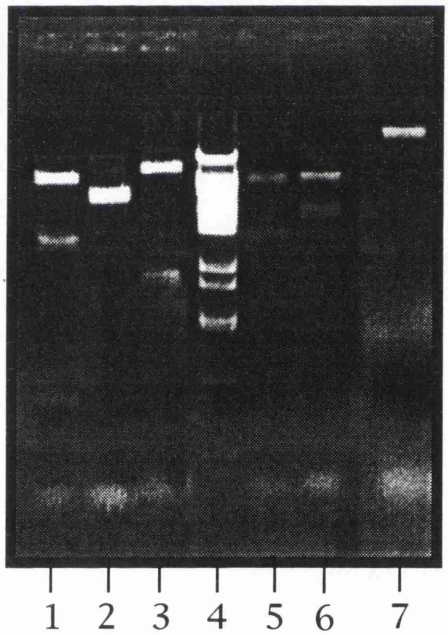


FIGURE 6.2a

Restriction digestion of LK444/GLUT 4 DNA

Colony 8 and colony 14 DNA was digested with 0.5-1.0 units of each restriction enzyme in a reaction volume of 20µl for 60 min at 37°C. 1µl of RNase was then added to each reaction and the samples were incubated for a further 30 min at 37°C. Following this, 5µl of 5X DNA loading buffer was added to each reaction. The samples were then electrophoresed on a 1% agarose gel in a buffer tank filled with 1 x TAE buffer containing 25µg/ml ethidium bromide. Electrophoresis was carried out until the dye front had migrated 2 thirds of the way along the gel and the DNA fragments were viewed under U.V. light. A photograph of the gel under U.V. light is shown below and the samples contained in each gel well are listed. The sizes of the DNA fragments generated from the *Bst E* II-digested λ ladder in base pairs are as follows; 8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264.

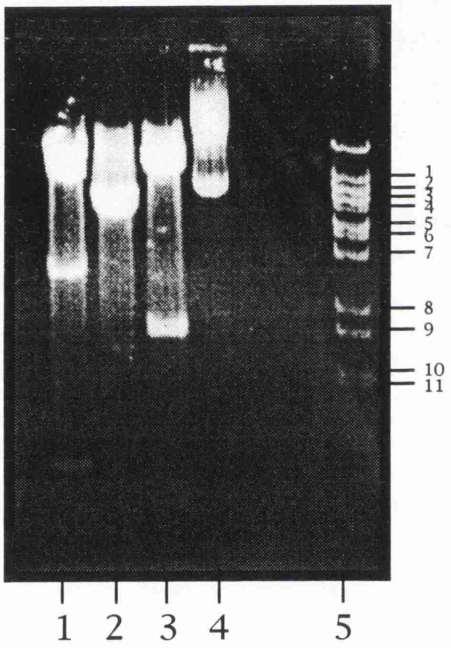


Lanes 1 Sample 14 digested with *Bam* HI/*Bgl* II
2 Sample 14 digested with *Eco* RI
3 Sample 14 digested with *Sal* I
4 *Bst E* II-digested λ ladder
5 Sample 8 digested with *Bam* HI/*Bgl* II
6 Sample 8 digested with *Eco* RI
7 Sample 8 digested with *Sal* I

FIGURE 6.2b

Restriction digestion of LK444/GLUT 4

Colony 14 DNA was digested for 60 min at 37°C with 0.5-1.0 units of each restriction enzyme in a reaction volume of 20µl. 1µl of RNase was then added to each reaction and the samples were incubated for a further 30 min at 37°C. Following this, 5µl of 5X DNA loading buffer was added and the samples were electrophoresed on a 1% agarose gel in a gel tank filled with 1 x TAE buffer containing 25µg/ml ethidium bromide. Electrophoresis was carried out until the dye front had migrated 2 thirds of the way along the gel and the DNA fragments were viewed under U.V. light. A photograph of the gel under U.V. light is shown below and the samples contained in each well are listed. Also listed are the sizes of the DNA fragments generated in the *Bst E* II-digested λ sample.



Lanes 1 Sample 14 digested with *Bam* HI/*Bgl* II
2 Sample 14 digested with *Eco* RI
3 Sample 14 digested with *Sal* I
4 Sample 14 digested with no enzyme
5 *Bst E* II-digested λ ladder

λ ladder DNA fragment sizes in base pairs are (1) 8454, (2) 7242, (3) 6369, (4) 5686, (5) 4822, (6) 4324, (7) 3675, (8) 2323 (9) 1929, (10) 1371 (11) 1264.

6.3.2 Immunoblotting of lysates prepared from GLUT 4-transfected H4IIe cells

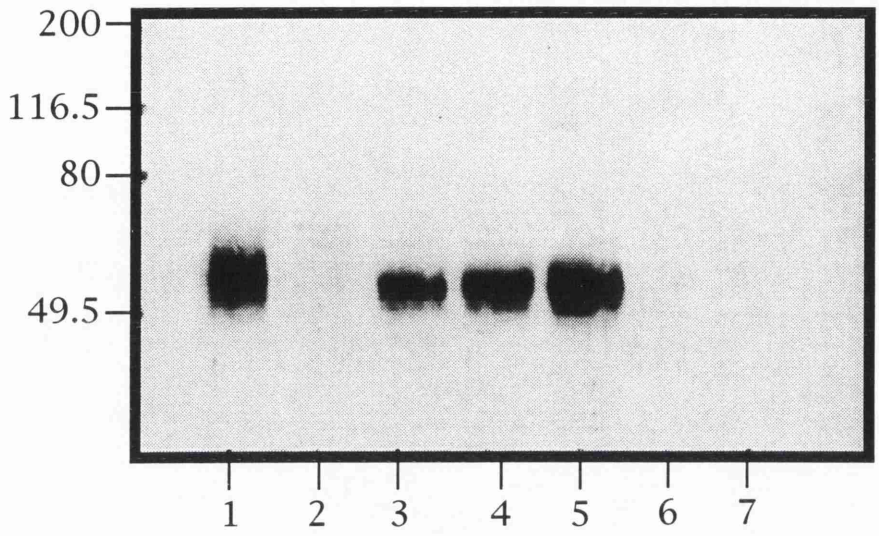
Immunoblotting of cell lysates prepared from several individual antibiotic-resistant clones with affinity-purified GLUT 4 antibodies identified a band at 50kDa in one clone designated 5A (Fig. 6.3). No immunoreactive band was detectable in lysates prepared from wild-type H4IIe cells or from a clone designated 3B. A band at 50kDa was also detectable in low density microsomal membranes prepared from 3T3-L1 adipocytes but not in membranes prepared from rat liver (Fig. 6.3). These data suggested that the immunoreactive band detected in the cell lysate prepared from 5A cells represented GLUT 4 protein. Immunoblots were developed using the ECL detection system which facilitated the detection of very low levels of the protein.

6.3.3 Quantitation of GLUT 4 in clone 5A cells using the ECL detection system and scanning densitometry

5µl (4-6µg protein) and 15µl (12-18µg protein) of lysate sample, prepared from clone 5A cells, were electrophoresed on 10% polyacrylamide gels and proteins transferred to nitrocellulose membranes (see Fig. 6.4). Membranes were then blocked in 5% non-fat milk/1st wash buffer and probed with anti-GLUT 4 antibodies (see 2.5.1). The immunoblots were then developed using the ECL detection system (see 2.6.2) and the immunoreactive bands generated quantitated by scanning densitometry (Fig. 6.4). GLUT 4 levels in clone 5A cell lysates were calculated relative to GLUT 4 standard samples in which the amount of GLUT 4 was known in ng per µl of sample. These membrane GLUT 4 standard samples were prepared from cells which had been stably transfected with GLUT 4 protein and were a gift from Dr. G I. Bell, University of Chicago. The amount of GLUT 4 protein expressed per

FIGURE 6.3

Immunological identification of GLUT 4 in GLUT 4-transfected H4IIe cell lysates

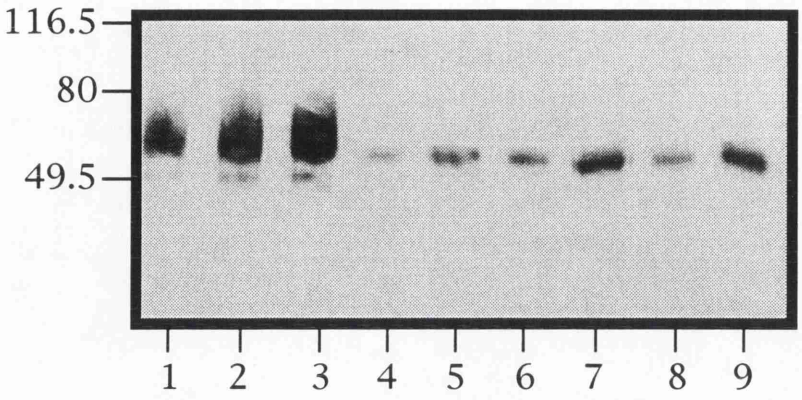


Cell lysates, prepared from clone 5A and clone 3B cells, were electrophoresed and transferred to nitrocellulose membranes as described in section 2.3 and 2.4. Nitrocellulose membranes were then probed with 10µg/ml affinity purified anti-GLUT 4 antibodies in 1% non fat milk/1st wash buffer, and the blots were developed using the ECL detection system. The positions of the molecular weight markers in kilodaltons are indicated to the left hand side of the immunoblot.

Samples contain 15µg basal 3T3-L1 low density microsomal membranes (lane 1), 5µg rat liver membranes (lane 2), 5µg 5A lysate (lane 3), 10µg 5A lysate (lane 4), 15µg 5A lysate (lane 5), 15µg 3B lysate (lane 6), 15µg wild-type H4IIe cell lysate (lane 7).

FIGURE 6.4

Immunological identification of GLUT 4 in H4IIe clone 5A lysate samples for GLUT 4 quantitation by scanning densitometry



Cell lysates, prepared from 3 independent preparations of clone 5A cells, were electrophoresed and transferred to nitrocellulose membranes (see 2.3, 2.4). Nitrocellulose membranes were then probed with 10µg/ml affinity purified anti-GLUT 4 antibodies in 1% non fat milk/1st wash buffer and the blots were developed using the ECL detection system. The amount of GLUT 4 was quantified by the densitomeric scanning of the immunoblot signals from the ECL developed autoradiograph. The positions of the molecular weight markers in kilodaltons are indicated to the left hand side of the immunoblot.

Samples contain 3ng GLUT 4 standards (lane 1), 5ng GLUT 4 standards (lane 2), 7ng GLUT 4 standards (lane 3), 4µg clone 5A lysate prep 1. (lane 4), 12µg clone 5A lysate prep 1. (lane 5), 6µg clone 5A lysate prep 2. (lane 6), 18µg clone 5A lysate prep 2. (lane 7), 6µg clone 5A lysate prep 3. (lane 8), 18µg clone 5A lysate prep 3. (lane 9).

μg of total protein in these cells was determined from GLUT 4-specific cytochalasin B-binding studies performed by Dr G. I. Bell.

Quantitation results indicated that clone 5A contained 57.6 ± 13 ng GLUT 4 per mg of total membrane protein.

6.3.4 [^{3}H] 2-deoxyglucose uptake in GLUT 4-transfected and non-transfected H4IIe cells

H4IIe cells were grown to confluency on 6-well plates and [^{3}H] 2-deoxyglucose uptake determined in both resting cells and in cells stimulated with 1μM insulin for 30 and 60 min. Uptake values were corrected for any non-specific association of the sugar with the cells by subtracting uptake values measured in the presence of 10μM cytochalasin B, a potent inhibitor of D-glucose transport. Assays were performed in triplicate for all clones and transport rates, determined from 3 independent experiments, are shown in Tables 6.2a and 6.2b and in Fig. 6.5. [^{3}H] 2-deoxyglucose uptake values were calculated in pmol/min/mg protein.

The results in Tables 6.2a and 6.2b show that, in resting cells, 2-deoxyglucose uptake rates were 146 ± 38 and 163 ± 30 pmol/min/mg for clones 5A and 3B respectively. Hence there was no significant difference in basal 2-deoxyglucose uptake in GLUT 4-expressing 5A cells relative to control 3B cells. Thus these data suggest that GLUT 4 protein did not contribute to basal 2-deoxyglucose uptake in resting clone 5A cells.

Stimulation of control 3B cells with 1μM insulin for 30 min and 60 min resulted in a 2.5 -fold increase in 2-deoxyglucose uptake relative to uptake observed in basal cells (Fig 6.5). Insulin stimulation for 30 min of 5A cells resulted in a 2.7 -fold increase in 2-deoxyglucose uptake relative to basal uptake, an increase similar to that observed in clone 3B cells stimulated with insulin for 30 min. However in contrast to results obtained in 3B cells, when clone 5A cells were stimulated with insulin for 60 min a 5.5 -fold increase over basal uptake was observed (Fig. 6.5).

TABLE 6.2

The tables below show [^{3}H] 2-deoxyglucose uptake measured in H4IIe clones 5A and 3B. H4IIe 5A and 3B cells were grown to confluency on 6-well plates and then incubated in the absence or presence, for 30 min, of 1μM insulin. Sugar uptake was initiated by the addition of 0.1mM [^{3}H] 2-deoxyglucose, 0.5μCi/ml (final concentrations) and transport was measured for 10 min. Non-specific [^{3}H] 2-deoxyglucose uptake was corrected for by subtracting uptake measured in the presence of 10μM cytochalasin B, a potent inhibitor of glucose transport. Assays were performed in triplicate for all incubation conditions and the transport rates expressed in pmol/million cells/mg protein, determined from 3 independent experiments, are shown below. The mean uptake values were also determined and results are shown below in bold lettering. A graphic representation of the mean values is shown in Fig.6.5

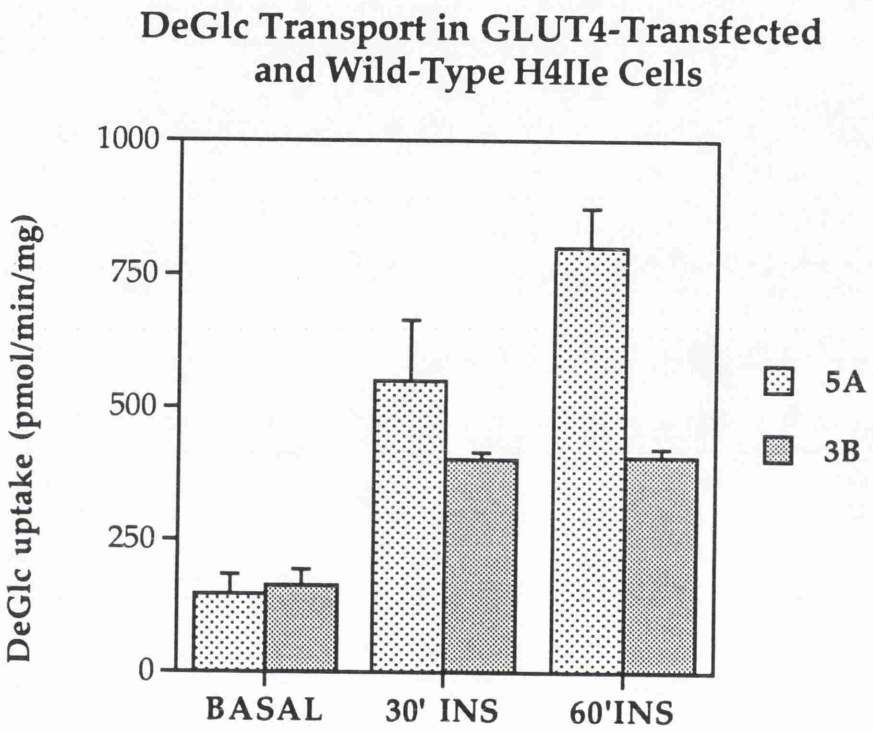
(a)

CLONE 5A	**Basal**	**30' Insulin**	**60' Insulin**
prep. 1	132 ± 32	440 ± 64	798 ± 168
prep. 2	115 ± 25	500 ± 61	732 ± 41
prep. 3	193 ± 19	709 ± 187	902 ± 103
Mean value	**147 ± 38**	**549 ± 115**	**800 ± 73**

(b)

CLONE 3B	**Basal**	**30' Insulin**	**60' Insulin**
prep. 1	150 ± 30	395 ± 50	408 ± 20
prep. 2	206 ±10	421 ± 5	426 ± 78
prep. 3	134 ± 74	391 ± 7	387 ± 57
Mean value	**163 ± 30**	**402 ± 13**	**407 ± 15**

FIGURE 6.5


DeGlc Transport in GLUT4-Transfected and Wild-Type H4IIe Cells
DeGlc uptake (pmol/min/mg)
1000
750
500
250
0
BASAL
30' INS
60'INS
5A
3B

Thus, in clone 5A cells, 60 min stimulation with insulin promoted a greater increase in 2-deoxyglucose uptake relative to that observed in control 3B cells under the same conditions. One explanation which could account for the 5.5 -fold increase in 2-deoxyglucose uptake observed in 5A cells could be that, in response to insulin, GLUT 4 protein levels increased at the plasma membrane. Consequently it could be hypothesised that insulin stimulation of 5A cells for 60 min resulted in the translocation of transfected-GLUT4 protein from an intracellular location to the plasma membrane.

The following part of this study was carried out in an attempt to determine whether GLUT 4 translocation did occur in 5A cells and could account for the increase in 2-deoxyglucose observed in response to insulin.

6.3.5 Immunoprecipitation of [^{3}H]-ATB-BMPA labelled GLUT 4 in basal H4IIe cells and cells stimulated with insulin for 60 min to demonstrate insulin-stimulated GLUT 4 translocation

H4IIe cells were incubated in the absence or presence, for 30 min, of 1μM insulin. Following this, [^{3}H]-ATB-BMPA label was added, and the cells were irradiated in a Rayonet reactor for 1 min. GLUT 4 protein was then immunoprecipitated with anti-GLUT 4 antibodies and the samples electrophoresed on large 12.5% polyacrylamide gels to determine if GLUT 4 labelling and immunoprecipitation of the labelled protein had occurred. Following electrophoresis, the gel lanes were sliced into 0.5cm sections and the positions of each gel slice, relative to the position of the molecular weight markers, was noted. The gel chips were then dried, solubilised and tritium incorporation determined by liquid scintillation counting.

It was expected that, in lanes containing GLUT 4-immunoprecipitated samples, an increase in tritium incorporation would be observed in gel chips isolated from the gel region corresponding to the 50kDa marker, since this region would

contain GLUT 4 protein. Moreover it was hoped that tritium incorporation would be greater, within the 50kDa region, in gel chips isolated from lanes loaded with immunoprecipitated GLUT 4 prepared from insulin-stimulated cells relative to lanes loaded with immunoprecipitated GLUT 4 prepared from basal cells. This would have demonstrated that more label had been incorporated into GLUT 4 protein at the cell surface in insulin-stimulated cells relative to basal cells and consequently that GLUT 4 translocation had occurred in response to insulin.

The results obtained however, did not fulfil these expectations. No increase in tritium incorporation was observed above the background level in gel chips isolated from the 50kDa region of lanes containing immunoprecipitated-GLUT 4 samples prepared from either basal or insulin-stimulated cells. These results suggested that either [^{3}H] ATB-BMPA labelling of GLUT 4 had been unsuccessful, or that GLUT 4 antibodies had failed to immunoprecipitate [^{3}H] ATB-BMPA-labelled GLUT 4 protein.

To determine whether the immunoprecipitation step had been successful, 5µl and 10µl of [^{3}H] ATB-BMPA-labelled immunoprecipitated GLUT 4 samples, prepared from basal and insulin stimulated cells, were subjected to electrophoresis on 10% SDS/polacrylamide gels. The proteins were transferred to nitrocellulose membranes and the blots probed with anti-GLUT 4 antibodies. The blots were then developed using ^{125}I-labelled goat anti-rabbit IgG and autoradiography.

Results obtained from the immunoblot autoradiographs were difficult to interpret however, since the signals generated by the heavy-light chain dimers and free heavy chains, originating from the GLUT 4 antibody used to immunoprecipitate the [^{3}H] ATB-BMPA-labelled GLUT 4, obscured the area of the film where the immunoprecipitated GLUT 4 protein was likely to be detected (data not shown). Therefore it was not possible to determine whether GLUT 4 immunoprecipitation had occurred. This problem could have been avoided however if [^{3}H] ATB-BMPA-labelled GLUT 4 protein had been immunoprecipitated from clone 5A cells using anti-GLUT 4 antibodies raised in a different species to the detection antibody. For

example anti-GLUT 4 antibodies raised in sheep would not have been recognised by the secondary ^{125}I-labelled goat anti-rabbit IgG antibodies used for blot development, hence only the immunoprecipitated GLUT 4 protein would have been detected after probing the nitrocellulose with rabbit anti-GLUT 4 antibodies, blot development and autoradiography. However, since anti-GLUT 4 antibodies raised in other species were not available at this time, this procedure could not be carried out.

Overall this [^{3}H] ATB-BMPA labelling procedure did not provide any data regarding potential insulin-stimulated GLUT 4 translocation in clone 5A cells.

6.3.6 Trypsinisation of cell surface GLUT 4 in clone 5A cells stimulated with insulin

A study carried out by Czech and Buxton showed that the appearance of GLUT 4 protein on the cell surface of rat adipocytes, following insulin stimulation, could be assessed using an approach independent of subcellular fractionation and cell surface glucose transporter labelling (Czech and Buxton, 1993).

These investigators proposed that, since GLUT 4 contains 2 putative trypsin cleavage sites in the exofacial loop, trypsin digestion of the protein on the cell surface of rat adipocytes would result in the generation of smaller molecular weight GLUT 4 species (Czech and Buxton, 1993). Therefore this approach was employed in the study described here in an attempt to demonstrate the potential translocation of GLUT 4 in clone 5A cells in response to insulin. Hence, if small molecular weight GLUT 4 species could be detected after trypsin treatment of clone 5A cells following insulin treatment, then this would demonstrate that GLUT 4 protein was present at the plasma membrane and consequently susceptible to trypsin digestion.

3T3-L1 adipocytes were used as control cells in this study since cell surface GLUT 4 protein levels are known to be increased following insulin stimulation (Zorzano *et al.*, 1989; Holman *et al.*, 1990; Calderhead *et al.*, 1990; Piper *et al.*, 1991; Slot *et al.*, 1991a). Therefore these cells were treated with trypsin, under the

conditions defined by Czech and Buxton (1993), to demonstrate that the protocol was a valid method to demonstrate GLUT 4 translocation.

Clone 5A cells and 3T3-L1 adipocytes were stimulated with 1μM insulin for 60 min and then incubated with 2mM sodium cyanide for 20 min to inhibit membrane protein recycling. Following this, cells were treated with trypsin for 15, 30 and 60 min to allow trypsin digestion of cell surface GLUT 4. The digestion reactions were quenched after the appropriate incubation times, the cells were washed with buffer containing trypsin inhibitor and then resuspended in 1X SDS Laemmli sample buffer containing 4mg/ml trypsin inhibitor and 20mM DTT. The samples were subsequently electrophoresed on 10% SDS/polyacrylamide gels and the proteins were transferred to nitrocellulose membranes. The membranes were then probed with anti-GLUT 4 antibodies and blots were developed using the ECL detection system to facilitate the detection of very low amounts of potentially trypsin-digested GLUT 4 protein.

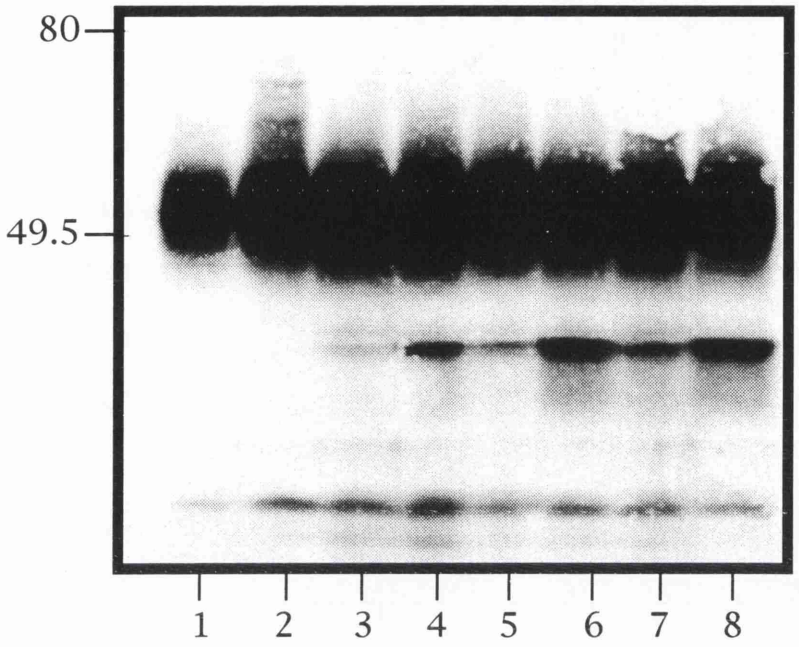
Results from clone 5A and 3T3-L1 immunoblots showed that a band at 50kDa was detectable in all samples (Fig. 6.7 and Fig. 6.8). This immunoreactive species was also detectable in plasma membranes prepared from insulin stimulated 3T3-L1 adipocytes. Therefore the immunoreactive band detected at 50kDa in all lanes probably represented intact GLUT 4 protein.

Results from 3T3-L1 immunoblotted samples (Fig. 6.7) suggested that trypsinisation of cell surface GLUT 4 had occurred in samples prepared from insulin-stimulated trypsin-treated cells. Immunoreactive bands at 27kDa were visually detectable in insulin-treated samples exposed to trypsin for 15, 30 and 60 min. The intensity of the lower immunoreactive band was greater in samples incubated with trypsin for increasing times. The intensity of the lower molecular weight immunoreactive species was greatest in the gel lane loaded with sample prepared from insulin-stimulated cells treated with trypsin for 60 min (Fig. 6.7).

Densitometric scanning of the immunoreactive bands was carried out in an attempt to assess the proportion of total GLUT 4 which had undergone trypsinisation (see discussion). Results in Table 6.3 show that, following insulin stimulation and

FIGURE 6.7

Proteolytic cleavage of cell surface GLUT 4 in control (bas) and insulin-treated (ins) 3T3-L1 adipocytes by trypsin in cyanide-poisoned 3T3-L1 adipocytes



Cell lysates, prepared from trypsin-digested 3T3-L1 adipocytes, were electrophoresed and transferred to nitrocellulose membranes (see 2.3, 2.4). Nitrocellulose membranes were then probed with 10μg/ml affinity purified anti-GLUT 4 antibodies in 1% non fat milk/1st wash buffer and the blots were developed using the ECL detection system. The signals from the immunoreactive bands (the 50kDa band and the lower molecular weight band at 27kDa) were quantitated by densitometric scanning (see table 6.3). The positions of the molecular weight markers in kilodaltons are shown to the left hand side of the immunoblot.

Samples contain bas 3T3-L1 no trypsin (lane 1), ins 3T3-L1 no trypsin (lane 2), bas 3T3-L1 15 min trypsin (lane 3) ins 3T3-L1 15 min trypsin (lane 4) bas 3T3-L1 30 min trypsin (lane 5) ins 3T3-L1 30 min trypsin (lane 6) bas 3T3-L1 60 min trypsin (lane 7) ins 3T3-L1 60 min trypsin (lane 8).

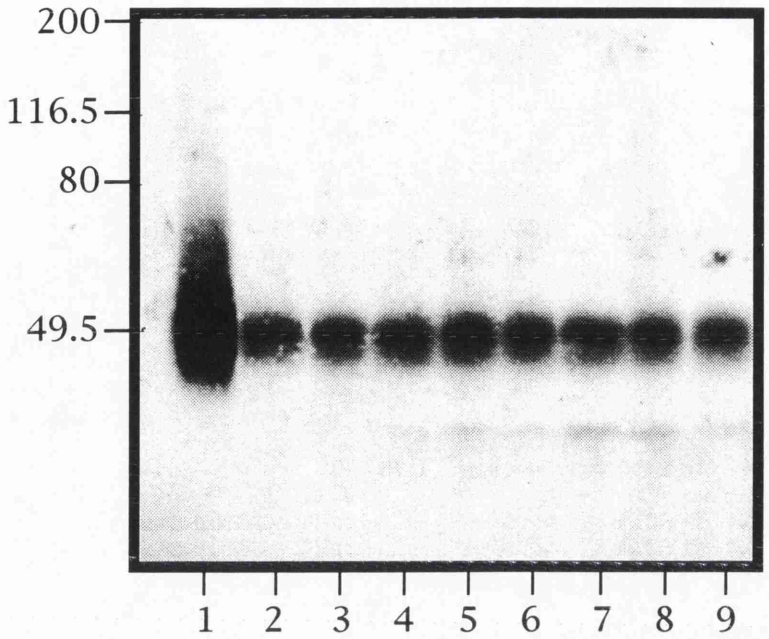
TABLE 6.3

Confluent 3T3-L1 adipocyte cells, grown on 6-well plates were incubated at 37°C in the absence and presence, for 30 min, of 1μM insulin. Cells were then treated with TPCK-treated trypsin for 15, 30 or 60 min at 37°C. The cells were then washed gently 3 times with 4ml of 2mg/ml soybean trypsin inhibitor in KRP, pH 7.4, and then pelleted at 1000rpm for 10 min. The cell pellets were then resuspended in 1X SDS sample buffer containing trypsin inhibitor. Samples were then electrophoresed on 12.5% SDS/polyacrylamide gels and proteins transferred to nitrocellulose membranes. The membranes were then probed with anti-GLUT 4 antibodies and the blots developed using the ECL detection system. GLUT 4 protein was quantified by densitometric scanning of the immunoblot signals from the ECL-developed autoradiographs. The results in the table below show the percentage of total cellular GLUT 4 which had undergone trypsinisation. Results were calculated by assessing the signal obtained in the lower molecular weight immunoreactive band relative to the signals obtained in both the lower weight and intact GLUT 4 immunoreactive band.

3T3-L1 SAMPLE	PERCENTAGE OF TOTAL GLUT 4 TRYPSINISED
Basal /no trypsin	0.14
Insulin /no trypsin	0.00
Basal/30' trypsin	1.60
Insulin/30'trypsin	2.80
Basal/60' trypsin	2.80
Insulin/60' trypsin	9.50

FIGURE 6.8

Proteolytic cleavage of cell surface GLUT 4 in control (bas) and insulin-treated (ins) H4IIe clone 5A cells by trypsin in cyanide-poisoned H4IIe clone 5A cells



Cell lysates, prepared from trypsin-digested H4IIe clone 5A cells, were electrophoresed and transferred to nitrocellulose membranes (see 2.3, 2.4). Nitrocellulose membranes were then probed with 10μg/ml affinity purified anti-GLUT 4 antibodies in 1% non fat milk/1st wash buffer and the blots were developed using the ECL detection system. The signals from the immunoreactive bands (the 50kDa band and the lower molecular weight band at 27kDa) were quantitated by densitometric scanning (see table 6.3). The positions of the molecular weight markers in kilodaltons are shown to the left hand side of the immunoblot.

Samples contain bas 3T3-L1 LDM (lane 1) bas clone 5A no trypsin (lane 2), ins clone 5A no trypsin (lane 3), bas clone 5A 15 min trypsin (lane 4), ins clone 5A 15 min trypsin (lane 5), bas clone 5A 30 min trypsin (lane 6), ins clone 5A 30 min trypsin (lane 7), bas clone 5A 60 min trypsin (lane 8), ins clone 5A 60 min trypsin (lane 9).

TABLE 6.4

Confluent clone 5A cells, grown on 6-well plates were incubated at 37°C in the absence and presence, for 30 min, of 1μM insulin. Cells were then treated with TPCK-treated trypsin for 15, 30 or 60 min at 37°C. The cells were then washed gently 3 times with 4ml of 2mg/ml soybean trypsin inhibitor in KRP, 7.4, and then pelleted at 1000rpm for 10 min. The cell pellets were then resuspended in 1X SDS sample buffer containing trypsin inhibitor. Samples were then electrophoresed on 12.5% SDS/polyacrylamide gels and proteins transferred to nitrocellulose membranes. The membranes were then probed with anti-GLUT 4 antibodies and the blots developed using the ECL detection system. GLUT 4 protein was quantified by densitometric scanning of the immunoblot signals from the ECL-developed autoradiographs. The results in the table below show the percentage of total cellular GLUT 4 which had undergone trypsinisation. Results were calculated by assessing the signal obtained in the lower molecular weight immunoreactive band relative to the signals obtained in both the lower weight and intact GLUT 4 immunoreactive band.

5A SAMPLE	PERCENTAGE OF TOTAL GLUT 4 TRYPSINISED
Basal /no trypsin	0.4
Insulin/no trypsin	0.0
Basal/30' trypsin	0.0
Insulin/30'trypsin	1.8
Basal/60' trypsin	1.5
Insulin/60' trypsin	4.9

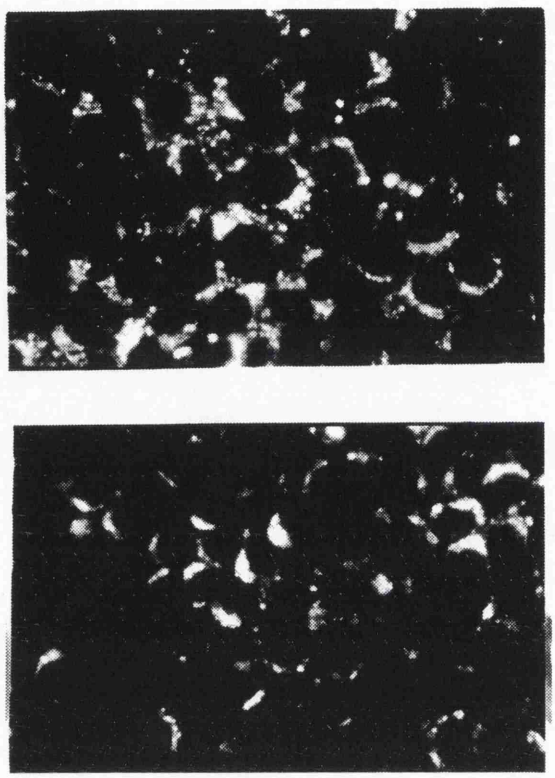
30 min of trypsin treatment, 2.8% of total GLUT 4 protein in 3T3-L1 adipocytes had been digested. The percentage of total GLUT 4 which had been trypsin-digested increased to 9.5% when cells were treated for 60 min with trypsin. Thus the presence of small molecular weight immunoreactive species suggested that GLUT 4 had undergone trypsin digestion.

Results from 5A immunoblotted samples (Fig. 6.8) showed that immunoreactive bands were detectable at 27kDa in samples prepared from insulin-stimulated cells treated with trypsin for 30 and 60 min. These results suggested that the bands of lower molecular weight detected by anti-GLUT 4 antibodies in insulin-stimulated, trypsin-treated cell samples represented products of digested cell surface GLUT 4. Consequently it could be postulated from these data that insulin stimulation promoted a small increase in cell surface GLUT 4 in clone 5A cells.

Densitometric scanning of the immunoreactive bands was carried out, as before, to determine the percentage of total GLUT 4 which had been trypsinised at the cell surface. Results, presented in Table 6.4, show that, after insulin stimulation and treatment with trypsin for 60 min, the lower weight immunoreactive band represented 4.9% of the total cellular GLUT 4 densitometry signal.

FIGURE 6.6a

Immunolocalisation of GLUT 4 protein in H4IIe clone 5A cells using anti-GLUT 4 antibodies and immunofluorescence



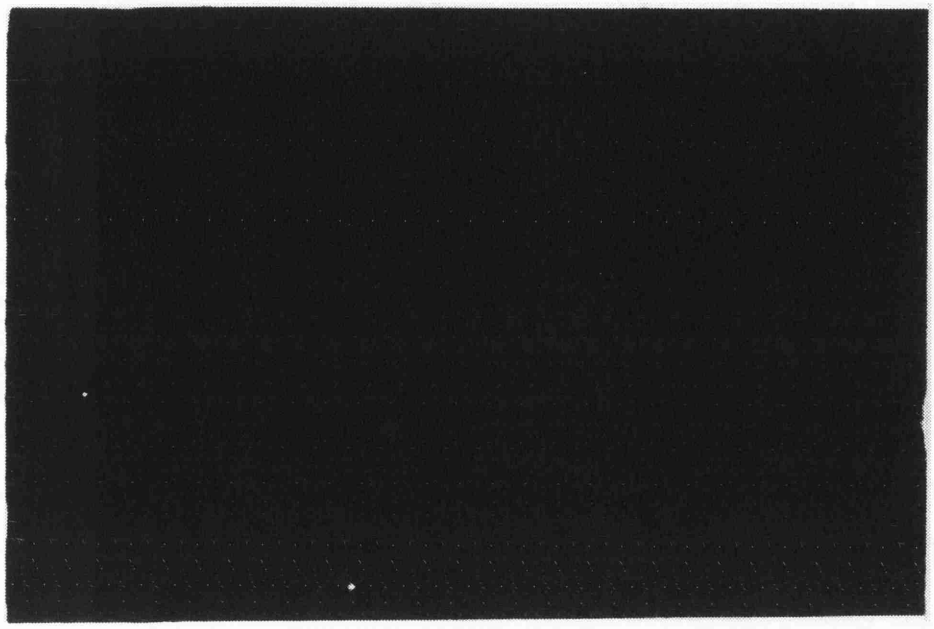
Panel a

Panel b

Clone 5A cells were incubated in the absence (panel A) or presence (panel B), for 30 min, of 1μM insulin. Cells were then methanol-fixed and incubated with Triton X-100 to permeabilise cell membranes. The cells were then incubated in the presence of affinity purified anti-GLUT 4 antibodies. Following this the cells were incubated in the presence of FITC-conjugated goat anti-rabbit IgG antibodies and the preparation was examined using a laser confocal immunofluorescence microscope. Photographs were taken of the preparations and these are shown here. Bright, white-coloured staining show areas of immunofluorescence and hence GLUT 4 localisation.

FIGURE 6.6b

Immunolocalisation of GLUT 4 protein in H4IIe clone 3B cells using anti-GLUT 4 antibodies and immunofluorescence



Clone 3B cells were incubated in the absence or presence, for 30 min, of 1μM insulin. Cells were methanol-fixed and incubated with Triton X-100 to permeabilise cell membranes. The cells were then incubated in the presence of affinity purified anti-GLUT 4 antibodies. Following this cells were incubated in the presence of FITC-conjugated goat anti-rabbit IgG antibodies and the preparation was examined using a laser confocal immunofluorescence microscope. The photograph shown here represents clone 3B cells incubated in the absence of insulin and labelled with affinity purified anti-GLUT 4 antibodies and FITC-conjugated goat anti-rabbit IgG antibodies.

6.4 DISCUSSION

This study was carried out to determine the insulin-responsiveness and contribution to cellular glucose uptake of GLUT 4, the insulin-sensitive glucose transporter, when expressed in a liver-type cellular environment. Hence this part of the investigation was the complement of the study carried out previously where 3T3-L1 cells were transfected with the liver cell transporter GLUT 2.

A common approach used to study GLUT 4 targeting and translocation in response to insulin has been to express GLUT 4 in cells which do not exhibit increased uptake in response to insulin (Hudson *et al.*, 1992; Shibasaki *et al.*, 1992). Results from such studies have indicated that GLUT 4 is sequestered to an intracellular region in transfected cells and cannot be recruited to the cell surface following insulin treatment. Hence it would seem that factors present in an adipocyte environment are necessary for the process of insulin-induced translocation and moreover that the expression of GLUT 4 protein alone does not confer insulin-sensitivity with regard to GLUT 4 translocation.

This study was carried out to determine if GLUT 4 protein, when expressed in a liver-type cell environment, could exhibit insulin-induced translocation and consequently if the factors necessary to induce GLUT 4 translocation were present in these cells. As mentioned previously H4IIe cells express a high number of insulin receptors (10,000-30,000 insulin receptors per cell) and are highly responsive to insulin. Studies have demonstrated that maximal stimulation of D-[U-^{14}C] glucose incorporation into glycogen occurs when cells are incubated in the presence of 10nM insulin (Hofmann *et al.*, 1980). In previous investigations H4IIe cells have proven useful as a hepatocyte model system in the study of insulin action (Hatada *et al.*, 1989). Therefore these insulin-sensitive cells were used here as a hepatocyte model system for performing transfection procedures and for studying glucose transport .

GLUT 4-expressing H4IIe clone 5A cells and non-GLUT 4 expressing clone 3B cells were assessed for their ability to transport 2-deoxyglucose in the absence

and presence of insulin. Results from such studies (Table 6.2 and Fig. 6.5) indicated that basal uptake was similar in both 5A and 3B cells suggesting that the expression of the additional GLUT 4 transporter in 5A cells did not contribute to basal uptake. These data alone suggested that GLUT 4 was not exposed at the cell surface and thus unable to contribute to 2-deoxyglucose uptake. Upon insulin stimulation for 30 min however, 2-deoxyglucose uptake was increased 2.5 and 2.7 -fold in clone 3B and 5A cells respectively. However upon 60 min insulin stimulation, uptake was increased 5.5 -fold over basal uptake in 5A cells. These results were in contrast to those observed in 3B cells where 60 min insulin stimulation only increased 2-deoxyglucose uptake 2.5 fold over basal uptake, an increase identical to that observed upon 30 min insulin stimulation. These results therefore suggested that the greater increase in insulin-stimulated uptake in 5A cells was a direct consequence of GLUT 4 expression.

A possible explanation for these observations could be that, upon insulin stimulation, transfected-GLUT 4 protein translocated from an intracellular region to the plasma membrane directly resulting in an increase in GLUT 4 at the plasma membrane and consequently an increase in 2-deoxyglucose uptake.

Various strategies were subsequently employed in the remaining part of the study in an attempt to demonstrate the putative increase in GLUT 4 protein at the plasma membrane in 5A cells, following insulin stimulation. As a first step, the membrane-impermeant glucose transporter-specific label, ATB-BMPA, was used to assess the levels of GLUT 4 protein at the cell surface in resting cells and in cells stimulated with insulin for 60 min. However, results indicated that, after immunoprecipitating GLUT 4 from basal and insulin-stimulated cells incubated in the presence of [^{3}H] ATB-BMPA label, no tritium incorporation was observed in any of the immunoprecipitated samples. These data suggested that either immunoprecipitation of GLUT 4 protein had been unsuccessful or that [^{3}H] ATB-BMPA labelling of GLUT 4 had not occurred.

Densitometric scanning of lysate samples prepared from GLUT 4-expressing clone 5A (Fig. 6.4) revealed that cells expressed 57.6 ± 13 ng GLUT 4 per mg of membrane protein. These levels were similar to the levels of GLUT 4 protein observed in 3T3-L1 adipocytes (30-54 ng GLUT 4 per mg of total protein :- Calderhead *et al.*, 1990). Therefore it was envisaged that GLUT 4 labelling should occur in clone 5A cells since [^{3}H] ATB-BMPA labelling of GLUT 4 in 3T3-L1 cells has been demonstrated previously (Yang *et al.*, 1992). However [^{3}H] ATB-BMPA GLUT 4 labelling could not be demonstrated in clone 5A cells. This was probably due to the fact that, although the level of GLUT 4 expression per mg of protein in clone 5A cells was similar to that observed in 3T3-L1 cells, the actual protein concentration per surface area was much less in clone 5A cells relative to 3T3-L1 adipocytes. Results from protein determination assays indicated that a 35mm plate of confluent 5A cells contained 150 to 250μg of protein. This was in marked contrast to protein concentrations determined for 3T3-L1 adipocytes which have been reported to be in the order of 1.5 mg of protein per 35mm plate (Calderhead *et al.* 1990). Therefore it could be suggested that [^{3}H] ATB-BMPA labelling of GLUT 4 protein in clone 5A cells was not observed since samples subjected to the labelling procedure contained too little protein or in other words insufficient GLUT 4 protein.

In a further attempt to demonstrate GLUT 4 translocation in response to insulin in 5A cells, Immunofluorescence techniques were employed. These investigations were carried out in collaboration with Banting and co-workers who used a secondary fluorescently-labelled antibody to label GLUT 4 antibody bound to its antigen. Results from these studies showed that, in basal cells, GLUT 4 staining was predominantly perinuclear and that immunofluorescent labelling was undetectable at the cell surface (Fig 6.6a) Following insulin stimulation and immunofluorescent staining however results were slightly more difficult to interpret. Immunofluorescent staining remained intense within perinuclear regions but a possible slight increase in immunofluorescence at the cell surface may have occurred (Fig. 6.6a). However this putative increase was difficult to see on the photographic

evidence shown here. Hence, since GLUT 4 immunofluorescence staining of 5A cells in the presence and absence of insulin was predominantly perinuclear, it could not be definitively demonstrated using this approach that GLUT 4 translocation occurred in response to insulin. As expected no immunofluorescent staining was observed in 3B cells incubated in the absence or presence of insulin since these cells did not express GLUT 4 protein (Fig 6.6b).

As a final approach to demonstrate the putative GLUT 4 translocation in 5A cells, a method developed by Czech and Buxton (1993) was employed. Cells were incubated in the presence or absence of insulin and then were treated with trypsin for 30 and 60 min. It was postulated that, since the exofacial loop of GLUT 4 protein encompasses two putative trypsin cleavage sequences, GLUT 4 protein at the surface of cells would be susceptible to digestion in the presence of trypsin. Hence trypsin digestion of GLUT 4 would result in the generation of GLUT 4-derived protein fragments. The rationale for utilising this approach in this study therefore was that in basal 5A cells little or no GLUT 4 would be available at the cell surface for trypsin digestion and hence little or no GLUT 4 digested fragments would be generated. If however, following insulin stimulation, GLUT 4 protein levels increased at the cell surface, the protein would be susceptible to trypsin digestion and small fragments of GLUT 4 would be generated. Using this method it was demonstrated that, in 3T3-L1 and 5A cells, low molecular weight protein species were detectable using anti-GLUT 4 antibodies in both basal and insulin-stimulated cells (Fig 6.7 and Fig. 6.8). The amount of GLUT 4 which was located at the cell surface and hence susceptible to trypsin digestion was then estimated by scanning densitometry. The immunoreactive band at 50kDa, representing intact GLUT 4 protein, and the lower molecular weight immunoreactive band representing trypsin-digested GLUT 4 were quantitated using this method. The amount of GLUT 4 which had been trypsinised was quantitated relative to total cell GLUT 4 protein and results are shown in Tables 6.3 and 6.4.

Results indicated that, in basal 3T3-L1 cells, very low levels of the low molecular weight immunoreactive species, representing trypsin-digested GLUT 4

protein, were detectable (Table 6.3). However, the immunodetectable signal from these species was very low indicating that only a small amount of GLUT 4 protein was present at the cell surface under basal conditions. Following 60 min insulin treatment and 60 min of trypsin digestion, the lower molecular weight immunoreactive band was visually greater in intensity relative to the corresponding band observed in basal cells. Densitometric scanning showed that the lower molecular weight species constituted 2.8% of total GLUT 4 protein in basal cells and 9.5% in cells stimulated with insulin for 60 min (Table 6.3). Hence after 60 min stimulation with insulin there was an approximate 6.7% increase in the amount of detectable low molecular weight GLUT 4 immunoreactive species and consequently an approximate 6.7% increase in cell surface GLUT 4 protein. Therefore it could be postulated that approximately 6.7% of the total cellular GLUT 4 protein had been recruited to the cell surface following insulin stimulation and was consequently susceptible to trypsin-digestion.

Results indicated that, in basal 5A cells, very low levels of the low molecular weight species, representing trypsin-digested GLUT 4 protein were detectable (Fig. 6.7). These results therefore indicated that only a small amount of GLUT 4 protein was present at the cell surface under basal conditions. However, following 60 min insulin treatment and 60 min of trypsin digestion, the lower molecular weight immunoreactive band was more visually intense relative to the corresponding band observed in basal cells. Densitometric scanning showed that the lower molecular weight species constituted 1.5% of total GLUT 4 protein in basal cells and 4.9% in cells stimulated with insulin for 60 min (Table 6.4). Hence after 60 min stimulation with insulin there was an approximate 3.4% increase in the amount of detectable low molecular weight GLUT 4 immunoreactive species and consequently an approximate 3.4% increase in cell surface GLUT 4 protein. Therefore it could be postulated that approximately 3.4% of the total cellular GLUT 4 protein had been recruited to the cell surface following insulin stimulation and was consequently susceptible to trypsin-digestion.

However, it should be noted that this relative quantitation method was inaccurate and consequently the assessment of the increase in cell surface GLUT 4 was only an estimate. The intensity of the low molecular weight immunoreactive bands was very low in both basal and insulin treated samples, hence measurements of their relative intensities could not be made very accurately.

A method frequently used to assess GLUT 4 translocation in 3T3-L1 adipocytes in response to insulin has involved preparing membrane fractions from basal and insulin stimulated cells and immunoblotting these fractions with anti-GLUT 4 antibodies. Hence, in a similar manner, the extent of GLUT 4 translocation in 5A cells following insulin stimulation could have been assessed by preparing membrane fractions from basal and insulin-stimulated cells and comparing the amount of GLUT 4 present in the isolated plasma membranes. However, a method for isolating pure membrane fractions from hepatoma cells has not yet been developed since the cells are very small. Therefore this method could not be employed to assess the potential insulin-induced translocation of GLUT 4 in 5A cells.

Results from 2-deoxyglucose uptake in 5A cells indicated that insulin stimulation resulted in a 5.5 -fold increase in 2-deoxyglucose uptake which may have resulted from the translocation of GLUT 4 protein from an intracellular region to the plasma membrane (Table 6.2a and Fig. 6.5). However, using the methodology available GLUT 4 translocation could not be definitively demonstrated in this study.

Results of immunofluorescence studies carried out on 5A cells by Banting and co-workers indicated that GLUT 4 protein was targeted to an intracellular region in GLUT 4 transfected H4IIe cells and did not undergo translocation in response to insulin. Hence these observations were in accordance with those reported in previous studies in which GLUT 4 was found to be intracellularly targeted when expressed in HepG2, CHO and 3T3-L1 fibroblast cells (Shibasaki *et al.* 1992; Hudson *et al.*, 1992; Robinson *et al.*, 1992). These investigators also found no evidence suggesting GLUT 4 translocation to the plasma membrane following insulin stimulation.

However, it should be emphasised that results obtained in this study from 2-deoxyglucose uptake measurements in basal and insulin-stimulated 5A cells suggested that a small degree of GLUT 4 translocation did occur. It was therefore disappointing that GLUT 4 translocation could not be conclusively demonstrated here.

It should be noted however that the 5.5 -fold increase in 2-deoxyglucose uptake observed in 5A cells following insulin stimulation may merely have occurred due to glucose transporter overexpression in H4IIe cells. Therefore, it would be interesting to determine whether a similar increase in insulin-stimulated 2-deoxyglucose uptake would be observed in H4IIe cells transfected with GLUT 2 protein. If a 5.5 -fold increase in 2-deoxyglucose uptake did occur in response to insulin in GLUT 2-transfected cells then it could be concluded that the insulin-stimulated increase in uptake was due to glucose transporter overexpression.

If however, 2-deoxyglucose uptake results in GLUT 2-transfected H4IIe cells in response to insulin were similar to those observed in control 3B cells then it cold be concluded that the insulin-stimulated increase in uptake observed in clone 5A cells was GLUT 4 specific.

Consequently, it will be important in future studies to assess the effect of GLUT 2 expression in H4IIe cells and in addition to find a method to demonstrate the putative insulin-induced GLUT 4 translocation in 5A cells. If GLUT 4 translocation could be demonstrated in 5A cells then this would represent the first demonstration of reconstituted insulin-stimulated translocation.

However, should results from future investigations prove that GLUT 4 translocation does not occur in 5A cells in response to insulin, then the findings from the study carried out here would lend further support to the hypothesis that insulin-induced GLUT 4 translocation requires factors specific to the adipocyte environment.

CHAPTER 7

DISCUSSION AND CONCLUSION

The initial part of this study was concerned with the distribution of facilitative glucose transporters in brain tissues. Meeting the energy requirements for oxidative metabolism in the mammalian brain is almost exclusively dependent on a sustained supply of glucose (Owen *et al.*, 1967; Lund-Anderson, 1979). Of the six major isoforms cloned, GLUT 1 has been described to constitute the blood-brain barrier glucose transporter mediating the transport of glucose from the circulation into the brain interstitium (Froehner *et al.,* 1988). However, RNA blotting studies have shown that in addition to GLUT 1, the GLUT 3 isoform is also expressed in the adult human, rabbit, monkey, rat, mouse (Yano *et al.*, 1991) and dog brain (Gerhart *et al.*, 1992).It was consequently suggested that GLUT 3 could be responsible for the transport of glucose across the plasma membrane of neuronal and glial cells. Previous studies in humans have shown that although GLUT 3 mRNA is detectable in a wide range of tissues, GLUT 3 protein levels are highest in the brain, with lower levels being detectable in heart, placenta and fat tissues (Shepherd *et al.*, 1992). Northern blotting studies in mouse tissues however, have shown that GLUT 3 mRNA has a more restricted tissue distribution in these species compared to humans and that it is expressed primarily in brain (Yano *et al.*, 1991).

The study carried out here was an attempt to determine whether the pattern of GLUT 3 protein expression in mice differed from that in humans and moreover whether it was in accordance with its more limited mRNA tissue distribution. Specific anti-peptide antibodies were generated against the COOH-terminal portion of mouse GLUT 3 which were shown to recognise GLUT 3 protein in mouse brain membranes at 50 kilodaltons. The immunoblot analysis of various other mouse tissue membranes;- 3T3-L1 murine adipocyte cell line membranes, murine liver and muscle membranes, was then carried out. Results however indicated that no GLUT 3 immunoreactivity was detectable in any of these tissues examined. The possibility

that the expression of GLUT 3 in mice was restricted to the brain and neurally derived tissues was subsequently examined. Immunoblotting data showed that GLUT 3 was expressed in murine NG 108 neuroblastoma x glioma cell membranes, but that no GLUT 3 immunoreactivity was detectable in the rat parental cell line. On the basis of these results it was suggested that GLUT 3 expression in humans and mice differs and that GLUT 3 expression may be controlled at different points during transcription and/or translation. Therefore it would seem that the role of GLUT 3 in the context of whole body glucose homeostasis may differ between species. However, with regard to glucose disposal in the brain GLUT 3 does seem to have an important role to play in both species. Further studies will be required to examine the precise location of GLUT 3 in neurons since it has been demonstrated that neurons are polarised cells where protein sorting can occur. Furthermore, it may be interesting to determine whether brain GLUT 3 mRNA or indeed protein levels are altered in diabetic or starved mice. It could be postulated that GLUT 3 and/or GLUT 1 expression would be up-regulated when blood glucose levels were very low, since the brain depends on glucose exclusively as an energy source.

Following on from this study, an investigation was then undertaken to determine the levels of GLUT 3 protein within different regions of rat brain. *In situ* hybridisation analysis of GLUT 3 in the mouse brain had previously indicated that the expression of this isoform was not homogeneous throughout all regions of the brain. After demonstrating that anti-GLUT 3 antibodies were able to cross-react with rat GLUT 3 protein, an examination of GLUT 3 protein levels in individually dissected regions of the rat brain was carried out. The levels of GLUT 1 protein were also determined since this transporter constitutes the blood-brain barrier transport protein (Froehner *et al.*, 1988). Results indicated that when GLUT 1 and GLUT 3 levels were expressed relative to the levels measured in the frontal cortex, the trend was such that GLUT 1 levels increased while GLUT 3 levels decreased towards the more posterior brain regions. It was therefore postulated that a low level of one transporter was compensated for by a higher expression of the other. Hence it could be suggested that the rates of transcription and translation of both transporters are

highly co-ordinated and that there may be "cross-talk" between factors which regulate the expression of each gene. In addition to determining the levels of GLUT 1 and GLUT 3 protein expression in different brain regions the possibility that other transporter isoforms could be expressed in particular regions was investigated. It has been reported previously that specific areas of the brain exhibit chemosensitivity and that their activity is influenced by glucose, free fatty acids, insulin and other endogenous metabolites (Oomura, 1976).Consequently it could be suggested that these areas of the brain express glucose transporters which can be regulated by such specific hormones and metabolites. The various brain regions were immunoblotted with specific anti-peptide antibodies to GLUT 2 and GLUT 4. Results indicated that GLUT 2 protein was detectable in all regions of the brain examined. These data were in contrast with those of Widmaier (1992) who found no evidence for the expression of pancreatic β-cell "glucose sensors" in rat brain. The brain can be likened to the pancreas in terms of its response and sensitivity to changes in glucose and metabolites of glucose. The difference however is that while glucose activates secretion in the pancreas it seems to be inhibitory for neurohormone release in the brain at similar concentrations (Widmaier *et al.* 1988). Therefore on the basis of the results obtained in this study it could be postulated that GLUT 2 in may be involved in the regulation of neurohormone secretion. However it should be noted that GLUT 2 immunoblots were developed using the ECL detection system therefore the level of GLUT 2 expressed was too low to be reliably quantitated. Further work will be required to characterise the potential role of GLUT 2 in glucose sensing in the brain and in addition those glucose concentrations which could activate neurohormone secretion. The most surprising and exciting data however was obtained when brain regions were probed for the expression of insulin-regulatable GLUT 4 protein. GLUT 4 protein, ordinarily expressed in insulin-sensitive tissues fat, heart and muscle was detectable in three regions of the brain;- the pituitary, cerebellum and hypothalamus. Both the pituitary and hypothalamus are regions which are located outside the blood-brain barrier therefore it is quite possible that these regions could be regulated or influenced by insulin since the hormone would not have to negotiate the

selectivity of the blood-brain barrier. It has been reported that specific regions of the hypothalamus possess specific insulin receptors (Havrankova *et al.*, 1978), therefore it could be hypothesised that insulin could regulate glucose uptake through the GLUT 4 glucose transporter. It should be noted however, that GLUT 4 immunoblots were developed using the ECL detection system and therefore the absolute amount of GLUT 4 protein could not be quantitated in the hypothalamus. It will be important in the future to determine the precise subcellular location of GLUT 4 in these brain regions and to determine whether translocation occurs in response to insulin, since in its native cell GLUT 4 translocates from an intracellular region to the plasma membrane following insulin stimulation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Zorzano *et al.*, 1989). It could be suggested that GLUT 4 protein would not be important for basal glucose uptake in these regions since they express high levels of GLUT 1 and GLUT 3. However it could be hypothesised that when glucose levels in the brain rise and insulin release is stimulated, GLUT 4 could be recruited to the cell surface and mediate an increase in glucose uptake in regions of the hypothalamus which express GLUT 4.

The main part of the investigation described herein was concerned with determining whether GLUT 2 and GLUT 4, when expressed in "foreign" cells maintained their native properties or whether their properties were influenced by their cellular environment. GLUT 2 and GLUT 4 are both facilitative-type glucose transporters which are expressed in insulin-responsive tissues. GLUT 2 is expressed in the liver where it mediates the bi-directional movement of glucose both into and out of hepatocytes. GLUT 4, in contrast is expressed in insulin-sensitive tissues fat, muscle and heart and, upon exposure to insulin moves from an intracellular region to the plasma membrane by a process termed translocation (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Slot *et al.*, 1991a) Although GLUT 2 and GLUT 4 are both expressed in insulin-responsive tissues, liver and fat respectively, insulin stimulation of resting hepatocytes does not stimulate glucose transport and, by inference GLUT 2 does not undergo insulin-induced translocation (Thorens *et al.*, 1988; Fukomoto *et al.*, 1988). The aim of this study was to determine whether

GLUT 2, when expressed in a fat cell environment could undergo translocation in response to insulin and complementary to this, whether GLUT 4 when expressed in a liver-type cell environment still exhibited translocation following insulin-stimulation. It was hoped that the results from such an investigation would help determine whether the specific properties of glucose transporter proteins are inherent in the protein sequence and/or whether cellular environment influences the properties of the individual transporters. Previous studies investigating the properties of GLUT 4 in GLUT 4-transfected cells have demonstrated that, although the protein is targeted correctly to intracellular membranes, the protein does not exhibit translocation to the plasma membrane following insulin stimulation (Shibasaki *et al.*, 1992). Consequently it has been postulated that GLUT 4 protein itself probably contains the information necessary for its correct targeting in cells, but that specific cellular factors, present in native GLUT 4-expressing cells are both necessary and essential for the translocation process.

GLUT 2 was expressed in the murine fibroblastic 3T3-L1 cell line which, following differentiation under defined conditions, exhibits the biochemical and morphological properties characteristic of mature fat cells (Green, 1978). Immunoblotting cell lysates prepared from a number of GLUT 2-transfected clones with anti-GLUT 2 antibodies revealed that GLUT 2 protein was expressed in two clones, designated 3T3/G2a and 3T3/G2b. Both of these clones expressed GLUT 2 protein at different levels, and it was hoped by using both clones in subsequent experiments that it would be possible to control for spurious results arising from GLUT 2 over-expression. Glucose and fructose uptake was determined in both clones and uptake values were compared to control-transfected and wild-type fibroblasts. The results for 2-deoxyglucose uptake revealed that basal uptake was higher in GLUT 2-expressing clones relative to control cells. These results suggested that the increased uptake observed in GLUT 2-expressing clones was attributable to the expression of the transfected transporter. It was postulated that the transfected protein was localised at the plasma membrane since it contributed to 2-deoxyglucose uptake under basal conditions. Insulin-stimulated 2-deoxyglucose uptake increased 2

-fold in both GLUT 2-expressing and control cells which suggested that GLUT 2 did not undergo translocation in response to insulin and that the insulin-stimulated increase in 2-deoxyglucose uptake was mediated by the endogenous GLUT 1 transporter. D-fructose uptake results indicated that basal uptake was significantly higher in GLUT 2-expressing cells and that uptake was highest in 3T3/G2a which expressed GLUT 2 at higher levels. Upon insulin stimulation, the fold increase in D-fructose uptake was greatest in fibroblast cells which expressed GLUT 2 at lower levels which suggested that a very small degree of GLUT 2 translocation may have occurred. Taken together these results suggested that GLUT 2 when expressed in fibroblasts did not translocate in response to insulin since D-fructose uptake was not significantly increased upon insulin exposure. In 3T3-L1 adipocytes insulin stimulation results in a 20 to 30 -fold increase in 2-deoxyglucose uptake due to the translocation of GLUT 4 protein. Insulin stimulation of GLUT 2-transfected fibroblasts resulted only in a very small increase (less than 2 -fold). Hence if translocation was occurring in GLUT 20-transfected cells then the extent of translocation was insignificant when compared to the extent of translocation observed in 3T3-L1 adipocytes.

The most important part of this study was concerned with assessing the effects of insulin on GLUT 2 when GLUT 2 was expressed in the differentiated cell, since in this situation the transfected protein would be expressed in a cell environment where glucose transport was highly insulin-regulated. Following the differentiation of GLUT 2-expressing 3T3-L1 cells to adipocytes, subcellular membrane fractions were prepared from basal and insulin-stimulated cells. These fractions were then immunoblotted with GLUT 2 and GLUT 4 antibodies to determine the levels of each transporter in low density microsomal fractions and plasma membrane fractions. Immunoblotting of samples prepared from GLUT 2-expressing clones 3T3/G2a and 3T3/G2b revealed that GLUT 2 was detectable in plasma membrane and low density microsomes, but that levels were highest in the plasma membrane. The levels of GLUT 2 detected in plasma membrane samples prepared from basal and insulin-stimulated 3T3/G2a cells indicated that GLUT 2 levels were similar which suggested

that GLUT 2 did not undergo translocation. Immunoblotting of subcellular fractions with anti-GLUT 4 antibodies revealed that in basal cells, a greater percentage of cellular GLUT 4 was associated with low density microsomes. Following insulin stimulation there was an increase in GLUT 4 at the plasma membrane with a concomitant decrease in the low density microsomal fractions in both GLUT 2-expressing clones and wild-type cells. Furthermore there was a similar amount of GLUT 4 increase at the plasma membrane in all cells which indicated that translocation seemed to be occurring to a similar extent in both GLUT 2-expressing and wild-type cells.

Results from D-fructose uptake assays indicated that uptake was increased two -fold over basal in 3T3/G2a cells. In wild-type adipocytes insulin stimulation results in a 20 to 30 -fold increase in glucose transport . Thus it would expected that if GLUT 2 translocated in response to insulin a comparable 20 to 30 -fold increase in D-fructose uptake would have been observed in GLUT 2-expressing cells. Hence on the basis of this data alone it would seem that insulin did not promote the translocation of GLUT 2 in adipocytes.

Results from 2-deoxyglucose uptake assays showed that compared to wild-type 3T3-L1 adipocytes, basal uptake was approximately 3 -fold higher in GLUT 2-expressing clones. These results were as expected since GLUT 2 immunoblot analysis of GLUT 2-expressing 3T3-L1 adipocyte membrane fractions had indicated that GLUT 2 was expressed predominantly at the plasma membrane and thus readily able to contribute to glucose uptake.

However, the insulin-stimulated rates of 2-deoxyglucose uptake observed in GLUT 2-expressing adipocytes were lower relative to the insulin-stimulated rates observed in wild-type cells. This decrease in insulin-stimulated 2-deoxyglucose uptake could not be explained by a down-regulation of GLUT 4 expression since immunoblotting of total cell membranes with anti-GLUT 4 antibodies revealed that levels of GLUT 4 were similar in both GLUT 2-expressing and wild-type cells.

The possibility that the lower rates of insulin-stimulated 2-deoxyglucose uptake in GLUT 2-expressing cells were due to glucose-induced" desensitisation"

were then investigated. As mentioned in greater detail in Chapter 5, Marshall *et al.* (1991) proposed that a unique metabolic pathway exists in adipocytes, the products of which lead to "insulin-resistance" (reduced sensitivity to insulin) by impairing insulin-induced glucose transporter translocation. This metabolic pathway, termed the hexosamine pathway, was proposed to be activated when the concentration of glucose in an adipocyte cell exceeds the major glucose utilising pathways such as glycolysis and the pentose-phosphate pathway. Results from studies by Marshall *et al.* (1991) showed that, in addition to glucose and insulin, glutamine was also required to induce desensitisation of the insulin-responsive transport system. The key role of glutamine appeared to be as a substrate for the first and rate-limiting enzyme of the pathway, glutamine-fructose 6-phosphate amidotransferase (GFAT), which utilises the amino group of the amino acid to convert fructose 6-phosphate to glucosamine 6-phosphate. Glucosamine itself was found to be a potent inducer of "insulin resistance" and moreover was found to induce desensitisation in the presence of GFAT inhibitors. Therefore it was concluded that glucosamine entered the hexosamine pathway at a point distal to GFAT amidation (see Fig. 5.6).

In the study carried out here, 2-deoxyglucose uptake was assessed in wild-type adipocytes and GLUT 2-expressing adipocytes after the cells were pre-incubated overnight with GFAT inhibitors. Results indicated however that GLUT 2-expressing adipocytes still exhibited reduced insulin-stimulated 2-deoxyglucose uptake relative to wild-type cells. On the basis of these findings it was suggested that the reduced sensitivity of GLUT 2-expressing cells could not be due to the activation of the hexosamine pathway. However it has been demonstrated very recently, by using the *Xenopus* oocyte expression system that GLUT 2 is able to transport glucosamine directly (Gould *et al.*, unpublished data). These investigations demonstrated that *Xenopus* oocytes microinjected with GLUT 2 mRNA were able to transport 2-deoxyglucose and that this uptake was reduced in the presence of increasing amounts of glucosamine. These data suggested that GLUT 2 was able to transport glucosamine directly, however further work will be carried out to characterise this transport more fully. Hence if these preliminary results are correct, then it could be

suggested that the expression of GLUT 2 in 3T3-L1 adipocytes leads to a reduction in insulin-stimulated 2-deoxyglucose uptake through the GLUT 2-mediated transport of glucosamine into the cell and the subsequent activation of the hexosamine pathway.

Alternatively it could be suggested that the expression of GLUT 2 protein in adipocytes disrupted normal GLUT 4 intracellular targeting by directing GLUT 4 protein to intracellular vesicles which were non-competent for translocation. Immunoblotting of subcellular membrane fractions prepared from basal and insulin-stimulated cells however, indicated that translocation occurred to the same extent in both GLUT 2-expressing and wild-type adipocytes. Hence these data suggested that there was no apparent impairment of GLUT 4 translocation in GLUT 2-expressing adipocytes. It should be noted however that GLUT 4 levels at the plasma membrane, and consequently the extent of translocation may have been over-estimated in GLUT 2-expressing cells. It could be suggested that a percentage of the GLUT 4 protein detected in the plasma membrane by Western blotting represented protein in the occluded state. Vannucci *et al.* (1992) reported that insulin-stimulated GLUT 4 transporters can exist in two distinct states within the adipocyte plasma membrane, one of which is functional and accessible to extracellular substrate, and one of which is non-functional and unable to bind extracellular substrate (in the occluded state). These investigators demonstrated that the functional and non-functional forms could be readily distinguished by ATB-BMPA labelling but not by Western blotting techniques, since ATB-BMPA only bound to those transporters exposed at the cell surface. It will be important in future studies to determine whether the reduction in insulin-stimulated glucose uptake observed in GLUT 2-expressing cells correlates with a reduction in ATB-BMPA-GLUT 4 labelling. In this way it should be possible to determine if the expression of GLUT 2 in adipocytes leads to a reduction in insulin-stimulated glucose transport activity by promoting a decrease in the number of functional GLUT 4 transporters at the cell surface.

It is not yet clear whether glucose transporters function as monomers or as oligomers in the intact cell membrane. However, evidence for the existence of

transporter oligomers in the membrane has been obtained by the expression of GLUT 1-GLUT 4 chimeric molecules in CHO cells (Pessino *et al.*, 1991). It could be postulated that the expression of GLUT 2 protein in adipocytes disrupted normal GLUT 4-GLUT 4 oligomer formation at the plasma membrane following insulin stimulation, and that consequently the number of functional GLUT 4 transporters was reduced. Thus the reduction in insulin-stimulated glucose transport in GLUT 2-expressing cells could have been attributed to the formation of non-functional GLUT 2-GLUT 4 oligomers. However, since little is known about transporter oligomerisation this suggestion is very speculative.

It would be interesting in the future to carry out further transfection studies in GLUT 2-expressing 3T3-L1 cells. It could be suggested that the hexosamine pathway could be inhibited by increasing the flux of glucose through the normal glucose utilising pathways. This could perhaps be achieved by transfecting GLUT 2-expressing cells with glucokinase cDNA. It could be suggested that a hexokinase with a higher K_m would work more efficiently in cells where the glucose flux was increased, and that consequently the phosphorylation of glucose would no longer be rate-limiting. Thus in GLUT 2-transfected cells expressing glucokinase, glucose would no longer have to be routed to alternative pathways.

Taken together the data from this part of the study suggest that perhaps both the glucose transport sequence influences glucose transporter targeting and insulin-responsiveness. It would seem that GLUT 2 protein in adipocytes was targeted to the same location as in its native hepatocyte cell i.e. at the plasma membrane. Therefore it could be suggested that this targeting information is inherent in the protein sequence. Furthermore, in an adipocyte cell environment GLUT 2 could not be induced to undergo insulin-stimulated translocation even although the native GLUT 4 transporter exhibited translocation. Hence, although the potentially necessary factors which may be intimately involved in GLUT 4 translocation were present in the adipocyte environment, GLUT 2 did not exhibit translocation in the same environment. Therefore again, this would suggest that the property of translocation resides in the protein sequence. However this suggestion is in disagreement with results from

previous studies which have shown that the expression of GLUT 4 alone does not confer insulin-responsive glucose transport (Shibasaki *et al.*, 1992; Hudson *et al.*, 1992). Results from these studies indicated that GLUT 4 was sequestered to an intracellular region in transfected CHO and NIH-3T3 cells but that it was not recruited to the cell surface following insulin treatment. However it should be stressed that in contrast to the study performed here, these studies were not carried out in insulin-sensitive cells.

The precise site for intracellular GLUT 4 sequestration in adipocytes remains to be defined. Therefore it could be postulated that GLUT 4 did not exhibit insulin-stimulated translocation in GLUT 2-transfected 3T3-L1 cells because it was targeted to an intracellular site different to that in wild-type adipocytes and that this site was insensitive to the effects of insulin.

The complement of this study, investigating the insulin responsiveness of GLUT 4 in a liver-type cell environment, indicated that relative to non-transfected cells, GLUT 4-expressing cells exhibited increased 2-deoxyglucose transport in response to insulin. Basal 2-deoxyglucose uptake was similar in both GLUT 4-expressing and non-GLUT 4 expressing cells which suggested that GLUT 4 was not targeted to the cell surface where it could readily contribute to basal glucose uptake. Various strategies were subsequently employed in the remaining part of the study in an attempt to demonstrate that the increase in 2-deoxyglucose uptake in GLUT 4-expressing cells was attributable to GLUT 4 translocation. Immunofluorescence techniques which are generally very sensitive in detecting low levels of protein provided no evidence to suggest that insulin promoted GLUT 4 translocation in GLUT 4-expressing cells. Results showed that GLUT 4 staining was predominantly perinuclear in both basal and insulin-stimulated cells. As a final attempt to demonstrate the putative insulin-induced translocation, a method developed by Czech and Buxton (1993) was employed. It was postulated that since the exofacial loop of GLUT 4 protein encompasses two putative trypsin cleavage sites, GLUT 4 protein at the surface of cells would be susceptible to digestion in the presence of trypsin. Hence trypsin digestion of GLUT 4 would result in the generation of GLUT 4-

derived protein fragments. GLUT 4-expressing cells were subsequently stimulated with insulin and subjected to trypsin treatment. Lysates were then prepared from the treated cells and the samples were immunoblotted with anti-GLUT 4 antibodies. Results indicated that low molecular weight species were detectable in cell lysates prepared from insulin-stimulated cells which had been treated with trypsin. However, since the low molecular weight species were very faint on the ECL-developed autoradiograph, GLUT 4 translocation could not be conclusively demonstrated. Therefore on the basis of these data, it could be suggested that translocation may not only be a property dictated by the protein sequence, but that it is also dependent on the specific cell environment.

It would appear from the results described herein that neither glucose transporter sequence or cellular environment exclusively dictates the ability of transporters to undergo insulin-induced translocation. Recent investigations have suggested that glucose transporter domain-domain interactions may be important in targeting GLUT 4 to an intracellular pool from which it may be recruited to the plasma membrane, and that translocation is restricted to the GLUT 4 species. Thus it would appear that sequences within the GLUT 4 molecule dictate targeting to a pool from which translocation may occur. However the preliminary results described herein using an insulin-responsive cell line (H4IIe) indicate that translocation of GLUT 4 may also occur in cells which do not normally exhibit this response, simply by expression of the GLUT 4 protein. Thus in conclusion, these data would suggest that translocation is dependent not only on the transporter isoform, but also on the cellular environment in which GLUT 4 is expressed, since other laboratories have shown that expression of GLUT 4 in (for example) fibroblastic cells does not render these cells insulin-responsive.

REFERENCES

Allard, W. J. and Lienhard, G. E. (1985) *J. Biol. Chem.* **260**, 8668-8675.

Asano, T., Takata, K., Katagiri, H., Tsukuda, K., Lin, J-L., Ishikara, H., Inukai, K., Hirano, H., Yazaki, Y. and Oka, Y. (1992) *J. Biol. Chem.* **267**, 19636-19641.

Axelrod, J. D. and Pilch, P. F. (1983) *Biochemistry*, **22**, 2222-2227.

Baldwin, S. A. (1993) *Biochim. Biophys. Acta.* **1154**, 17-49.

Baldwin, S. A., Baldwin, J. M., and Lienhard, G. E. (1982), *Biochem. J.* **21** 3836-3842

Baur, H. and Heldt, H. W. (1977) *Eur. J. Biochem.* **74**, 397-403.

Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukomoto, H., and Seino, S. (1990) *Diabetes Care* **13**, 13276-13282.

Birnbaum, M. J. (1989) *Cell* **57**, 305-315.

Birnbaum, M. J., Haspel, H. C., and Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5784-5788.

Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W. and Geuze, H. J. (1988) *J. Cell. Biol.* **106**, 69-76.

Boado, R. J. and Pardridge, W. M.(1990) *Biochem. Biophys. Res. Commun.* **166**, 174-179.

Brant, A. M., Jess, T. J., Milligan, G., Brown, C. M., and Gould, G. W. (1993) *Biochem. Biophys. Res. Commun.* **192**, 1297-1302.

Burant, C. F., Takeda, J., Brot-Laroche, E., Bell, G. I. and Davidson, N. O. (1992) *J. Biol. Chem.* **267**, 14523-14526

Cain, C. C., Trimble, W. S. and Lienhard, G. E. (1992) *J. Biol. Chem.* **267**, 11681-11684.

Cairns, M. T., Alvarez, J., Panico, M. Gibbs, A. F., Morris, H. R., Chapman, D. and Baldwin, S. A. (1987) *Biochim. Biophys. Acta.* **905**, 295-310.

Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D. and Lienhard, G. E. (1990) *J. Biol. Chem.* **265**, 13800-13808.

Carter-Su, C., Pessin, J. E., Mora, R., Gitomer, W. and Czech, M. P.(1982) *J. Biol. Chem.* **257**, 5419-5425.

Cepko, C. I., Roberts, B. E. and Mulligan, R. C. (1984) *Cell* **37**, 1053-1062

Charron, M. J., Brosius, F. C., Alper, S. L. and Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2535-2539.

Ciaraldi T. P., Horuh, R. and Metthaei, S. (1986) *Biochem. J.* **240**, 115-123.

Clarke, D. D., Lajtha, A. L. and Maker, H. S. (1981) In: *Basic Neurochemistry* 4th edn, ed. Seigal, G. Agaranoff, B., Albers, R. W., Molinoff, P. (Raven press, New York) pp. 541-564.

Coleman, D. C. (1978) *Diabetologia* **14**, 141-148.

Colville, C. A., Seatter, M. J., Jess, T. J., Gould, G. W. and Thomas, H. M. (1993) *Biochem. J.* **290**, 701-706.

Craik, J. D. and Elliott, K. R. F. (1979) *Biochem. J.* **182**, 503-508.

Cudd, A. and Nicolau, C. (1984) In: *Liposome Technology* ed. Gregoriadus, G. (CRC, Boca Raton, FL.) pp 207-221.

Cushman, S. W. and Wardzala, L. J. (1980) *J. Biol. Chem.* **255**, 4758-4762.

Czech, M. P. and Buxton, J. M. (1993) *J. Biol. Chem.* **268**, 9187-9190.

De Camilli, P. and Jahn, R. (1990) *Annu. Rev. Physiol.* **52**, 625-645.

deHerreros, A. G. and Birnbaum, M. J. (1989) *J. Biol. Chem.* **264**, 19994-19999.

Deziel, M. Pegg, N., Mack, E., Rothstein, A. and Klip, A. (1984) *Biochim. Biophys. Acta.* **772**,403-406.

Dohm, G. L., Tapscott, E. B., Pories, W. J., Dabbs, D. J., Flickinger, E. G., Meelheim, P., Fushiki, T., Atkinson, S. M., Elton, C. W. and Caro, J. F. (1988) *J. Clin. Invest.* **82,** 486-494.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. U.S.A* **84**, 74137417.

Flier, J. S., Mueckler, M., McCall, A. L. and Lodish, H. F. (1987) *J. Clin. Invest.* **79,** 657661.

Froehner, S. C., Davies, A., Baldwin, S. A. and Lienhard, G. E. (1988) *J. Neurocyt.* **17**, 173-178.

Frost, S. C. and Lane, M. D. (1985) *J. Biol. Chem.* **260**, 2646-2652.

Fukomoto, H., Kayano, T., Buse, J. B., Edwards, Y., Pilch, P. F., Bell, G. I. and Seino, S.(1989) *J. Biol. Chem.* **264**, 7776-7779.

Fukomoto, H., Seino, S., Imura, H., Seino, Y., Eddy, R. L., Fukushima, Y., Byers, M. G., Shows, T. B. and Bell, G. I. (1988) *Proc. Natl. Acad. Sci. U.S.A* **.85**, 5434-5438.

Garcia, J. C., Strube, M., Liengang, K., Keller, K. and Mueckler, M. (1992) *J. Biol. Chem.* **267**, 7770-7776

Gerhart, D. Z., Broderius, M. A., Borson, N. D. and Drewes, L. R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 733-737.

Gerhart, D. Z., LeVasseur, R. J., Broderius, M. A. and Drewes, L. R. (1989) *J. Neurosci. Res.* **22**, 464-472.

Gibbs, E. M., Lienhard, G E. and Gould, G. W. (1988) *Biochemistry* **27**, 6681-6685.

Goresky, C. A. (1967) In: *Medical Physiology* ed. Bergner, P. E. and Lashbaugh, C.C. pp 423-435.

Gorga, F. R., Baldwin, S. A. and Lienhard, G. E. (1979) *Biochem. Biophys. Res. Commun.* **91**, 995-961.

Gould, G. W. and Bell, G. I. (1990) *Trends in Biochem. Sci.* **15**, 18-23.

Gould, G.W. and Holman, G. D. (1993) *Biochem. J.* **295**, 329-341.

Gould, G. W., Derechrin, V., James, D. E., Tjordman, K., Ahern, S., Gibbs, E. M., Lienhard, G. E. and Mueckler, M. (1989) *J. Biol. Chem.* **264**, 2180-2184.

Gould, G. W., Thomas, H. M., Jess, T. J. and Bell, G. I. (1991) *Biochem. J.* **30**, 5139 5145.

Graessmann, M. and Graessmann, A. (1986) In: *Microinjection and Organelle Transplantation Techniques* ed. Celis, J. E., Graessman, A., Loyter, A. (Academic London), pp. 3-13.

Graham, F. L. and Van der Eb, A. J. (1973) *Virology* **52**, 456-467.

Green, H. (1978) *10th Miami Winter Symposium on Differentiation and Development*,ed. Ahmad, F., Schultz, J. Russel, T. R., Werner, R. (Academic press, New York) pp. 13-33.

Green, H. and Kehinde, O. (1974) *Cell* **1**, 113-116.

Green, H. and Kehinde, O. (1975) *Cell* **5**, 19-27.

Green, H. and Kehinde, O. (1976) *Cell* **7**, 105-113.

Grossbard, L. and Schimke, R. T. (1966) *J. Biol. Chem.* **241**, 3546-3560.

Gunning, P., Laevitt, J. Muscat, G., Sun-Yu, N. and Kedes, L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4831-4835

Hacker, H. J., Thorens, B., Grobholtz, R. (1991) *Histochemistry* **96**, 435-439.

Hamprecht, B., Glasser, T., Reisser, G., Bayer, E. and Propst, F. (1985) *Arch. Biochem. Biophys.* **268**, 264-275.

Haney, P. M., Slot, J. W., Piper, R. C., James, D. E., Mueckler, M. (1991) *J. Cell. Biol.***114**, 689-699.

Harik, S. I., Kalaria, R. N., Whitney, P. M., Andersson, L., Lundahl, P., Ledbetter, S. R. and Perry, G. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4261-4264.

Hatada, E. N., McClain, D. A., Potter, E., Ullrich, A. and Olefsky, J. M. (1989) *J. Biol. Chem.* **264**, 6741-6747.

Havrankova, J., Roth, J. and Brownstein, H. (1978) *Nature.* **272**, 827-829.

Hirshman, M. F., Goodyear, L. J., Wardzala, L. J., Horton, E. D., and Horton, E. S. (1990) *J. Biol. Chem.* **265**, 987-991.

Hofmann, C., Marsh, J. W., Miller, B. and Steiner, D. F. (1980) *Diabetes.* **29**, 865-874.

Holman, G. D., Kozka, I. J., Clark, A. E., Flower, C. J., Saltis, J., Habberfield, A. D., Simpson, I. A. and Cushman, S. W. (1990) *J. Biol. Chem.* **265**, 18172-18179.

Hopfer, U. (1987) *Physiol. Gastrointest. Tract*, 2nd edn. ed. Johnson, L. R. (Raven Press, New York) pp. 1499-1526.

Huang, S. C., Phelps, M. E., Hoffman, E. J., Sideris, K., Selin, C. J. and Kuhl, D. E. (1980) *Am. J. Physiol.* **238**, E69-E82.

Hudson, A. M., Ruiz, M. and Birnbaum, M. J. (1992) *J. Cell. Biol.* **116**, 785-797.

Iynedjian, P. B. (1993) *Biochem. J.* 293, 1-13.

Iynedjian, P. B., Pilot, P - R., Nouspikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ucla, C., Newgard, C B. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7838-7842.

James, D. E., Brown, R., Navarro, J., and Pilch, P. F. (1988) *Nature.* **333**, 183-185.

James, D. E. and Piper, R. C. (1993) *J. Cell. Sci.* **104**, 607-612.

James, D. E., Strube, M. and Mueckler, M. (1989) *Nature.* **338**, 83-87.

Jhun, B. H., Rampal, A. L., Liu, H., Lachaal, M. and Jung, C. Y. (1992) *J. Biol. Chem.* **267**, 17710-17715.

Jing, S., Spencer, T., Miller, K., Hopkins, C. and Trowbridge, I. S. (1990) *J. Cell. Biol.* **110**, 283-294.

Kadekaro, M., Timo-Iaria, C. and Vicentini, M. L. M. (1980) *J. Physiol.* **299**, 397-407.

Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. H., Cornelius, P., Pekala, P. H. and Lane, M. D. (1989) *Proc. Natl. Acad. Sci. U.S.A* . **86**, 3150-3154.

Kasanicki, M. A., Jessen, K. R., Baldwin, S. A., Boyle, J. M., Davies, A. and Gardiner, R. M. (1989) *Histochem. J.* **21**, 47-51.

Katagiri, H., Asano, T., Shibasaki, Y., Lin, J - L.,Tsukuda, K., Ishihara, H., Akanuma, Y., Takaku, F. and Oka, Y. (1991) *J. Biol. Chem.* **266**, 7769-7773.

Katzen, H. M. (1967) *Adv. Enzyme Regul.* **5**, 335-356.

Katzen, H. M., Soderman, D. D., Cirillo, V. J. (1968) *Ann. N.Y. Acad. Sci.* **151**, 351-358.

Kayano, T., Burant, C. F., Fukomoto, H., Gould, G. W., Fan, Y., Eddy, R. L., Byers, M.G., Shows, T. B., Seino, S. and Bell, G. I. (1990) *J. Biol. Chem.* **265**, 13276-13282.

Kayano, T., Fukomoto, H., Eddy, R. L., Fan, Y.-S., Byers, M. G., Shows, T. B. and Bell, G. I. (1988) *J. Biol. Chem.* **263**, 15245-15248.

Keller, K., Strube, M., Mueckler, M. (1989) *J. Biol. Chem.* **264**, 18884-18889.

Klip, A. and Marette, A. (1992) *J. Cell. Biochem.* **48**, 51-60.

Klip, A., Marette, A., Dimitrakoudis, D., Rampal, T., Gicca, A., Shi, Z - Q. and Vranic, M. (1992) *Diabetes Care* **15**, 1727-1776.

Klip, M., Rampal, T., Young, D. and Holloszy, J. O. (1987) *FEBS. Lett.* **224**, 224-230.

Laemmli, U. K. (1970) *Nature.* **227**, 680-685.

Laurie, S. M., Cain, C. C., Lienhard, G. E., Castle, J. D. (1993) *J. Biol. Chem.* **268**, 19110-19117.

Lerner, R. A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 3403-3407.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.

Loyter, A., Scangos, G. A., Ruddle, F. H. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 422-426.

Lund, S., Holman, G. D., Schmitz, O. and Pederson, O. (1993) *FEBS Lett.* **330**, 312-318.

Lund-Anderson, H. (1979) *Physiol. Rev.* **59**, 305-352.

Mackall, J. C., Student, A. K., Polakis, S. E. and Lane, M. D. (1976) *J. Biol. Chem.* **251**, 6462-6464.

Maenz, D. D. and Cheeseman, C. I. (1987) *J. Mem. Biol.* **97**, 259-266.

Magnuson, M. A.and Shelton, K. D. (1989) *J. Biol. Chem.* **264**, 15936-15942.

Maher, F.,Vannucci, S., Takeda, J. and Simpson, I. (1992) *Biochem. Biophys. Res Commun.* **182**, 703-711.

Mantych, G. J., James, D. E., Chung, H. D. and DeVaskar, S. U. (1992) *Endocrin.* **131**, 1270-1278.

Mantych, G. J., James, D. E. and DeVaskar, S. U. (1993) *Endocrin.* **132**, 35-40.

Marchmont, R. J., Ayad, S. R. and Houslay, M. D. (1981) *Biochem. J.* **195**, 645-652.

Marshall, S., Bacote, V. and Traxinger, R. R. (1991) *J. Biol. Chem.* **266**, 4706-4712.

Masters B. A., Shemer, J., Judkins, J. H., Clarke, D. W., Le Roith, D. and Raizada, M. K. (1987) *Brain Res.* **417**, 247-256.

Meglasson, M. D. and Matschinsky, F. M. (1984) *Am. J. Physiol.* **246**, E1-E3.

Merrall, N. W., Wakelam, M. J. O. , Plevin, R. and Gould, G. W. (1993) *Biochim. Biophys. Acta.* **1177**, 191-198.

Mizuno, Y. and Oomura, Y. (1984) *Brain Res.* **307**, 109-116.

Mueckler, M. Caruso, C., Baldwin, S. A., Panico, M., Blench, M., Morris, H. R., Allard,W. J., Lienhard, G. E., Lodish, H. F. (1985) *Science* **229**, 941-945.

Nagamatsu, S., Kornhauser, J. M., Burant, C. F., Seino, S., Mayo, K. E. and Bell, G. I. (1992) *J. Biol. Chem.* **267**, 467-472.

Navone, F., Jahn, R., Di Gioia, G., Stukenbrok, H., Greengard, P. and De Camilli, P. (1986) *J. Cell. Biol.* **103**, 2511-2527.

Neumann, E., Schaefer-Ridder, M., Wang, Y and Hofschneider, P. H. (1982) *EMBO J.* **1**, 841-845.

Oomura, Y. (1976) In *Hunger : Basic Mechanism and Clinical Implications* ed. Novin, D., Wyrwicka, W., Bray, G. A. (Raven press, New York) pp. 145-157.

Orci, L., Thorens, B., Ravazzola, M., Lodish, H. F. (1989) *Science.* **245**, 295-297.

Owen, D. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G. and Cahill, G.F. (1967) *J. Clin. Invest.* **46**, 1589-1599.

Palfreyman, R. W., Clark, A. E., Denton, R. M., Holman, G. D. and Kozka, I. J. (1992) *Biochem. J.* **284**, 275-281.

Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346-356.

Pessino, A., Hebert, D. N., Wonn, C. W., Harrison, S. A., Clancy, B. M., Buxton, J. M., Carruthers, A. and Czech, M. P. (1991) *J. Biol. Chem.* **266**, 20213-20217.

Phelps, M. E., Huang, S. C., Hoffman, E. J., Selin, C., Sokoloff, L. and Kuhl, D. E. (1976) *Ann. Neurol.* **6**, 371-388.

Pilkis, S. J., Exton, J. H., Johnson, R. A.and Park, C. R. (1974) *Biochim. Biophys. Acta.* **343**, 250-267.

Piper, R. C., Hess, L. J. and James, D. E. (1991) *Am. J. Physiol.* **260**, C570-C580.

Piper, R. C., Tai, C., Kulesza, P., Pang, S., Warnock, D., Baenziger, J., Slot, J. W., Geuze, H. J., Puri, C. and James, D. E. (1993) *J. Cell. Biol.* **121**, 1221-1232.

Piper, R. C., Tai, C., Slot, J. W., Hahn, C. S., Rice, C. M., Huang, H. and James, D. E. (1992) *J. Cell. Biol.* **117**, 729-743.

Pitot, H. C., Peraino, C., Morse, P. A. J. and Potter, V. R. (1964) *Natl. Cancer. Inst. Monogr.* **13**, 229-245.

Ploug, T., Galbo, H., Vinten, J., Jorgenson, M. and Richter, E. A. (1987) *Am. J. Physiol.* **253**, E12-E20.

Resh, M. D. (1982), *J. Biol. Chem.* **257**, 6987-6986.

Rhoads, D. B., Takano, Gattoni-Celli, S., Chen, C - C. and Isselbacher, K. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 9042-9046.

Ritter, R. C., Slusser, P. G. and Stone, S. (1981) *Science* **213**, 451-453.

Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992) *J. Cell. Biol.* **117**, 1181-1196.

Rosen, O. M., Smith, C. J., Fung, C. and Rubin, C. S. (1978) *J. Biol. Chem.* **253**, 7579-7583.

Rubin, C. S., Hirsch, A., Fung, C. and Rosen, O. M. (1978) *J. Biol. Chem.* **253**, 7570 7583.

Rubin, C. S., Lai, E. and Rosen, O. M. (1977) *J. Biol. Chem.* **252**, 3554-3557.

Sacca, L. (1987) *Diabetes/Meta. Rev.* **3**, 207-229.

Saller, C. F. and Chiodo, L. A. (1980) *Science* **210**, 1269-1271.

Schaffer, W. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 2163-2167.

Shanahan, M. F. (1983) *Biochemistry*, **22**, 2750-2756.

Shanahan, M. F. (1982) *J. Biol. Chem.* **257**, 7290-7298.

Shepherd, P. R., Gibbs, E. M., Wesslau, C., Gould, G. W. and Kahn, B. B. (1992) *Diabetes*, **41**, 1360-1364.

Shepherd, P. R., Gould, G. W., Colville, C. A., McCoid, S. C., Gibbs, E. M. and Kahn, B.B. (1992) *Biochem. Biophys. Res. Commun.* **166**, 174-179.

Shibasaki, Y., Asano, T., Lin, J.-L., Tsukuda, K., Katagari, H., Ishihara, H., Yazaki, Y. and Oka, Y. (1992) *Biochem. J.* **281**, 829-834.

Simpson, I. A. and Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55**, 1059-1089.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991a) *J. Cell. Biol.* **113**, 123-135.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991b) *Proc. Natl. Acad. Sci. U.S.A* .**88**, 7815-7819.

Sochor, M., Baquer, N. Z., Hothersall, J. S., McLean, P. (1990) *Biochem. Int.* **22**, 467-474.

Suzuki, K. and Kono, T. (1980) *Proc. Natl. Acad.Sci. U.S.A* .**77**, 2542-2545.

Thorens, B., Cheng, Z.-Q., Brown, D. and Lodish, H. F. (1990) *Am. J. Physiol.* **259**, C279-C285.

Thorens, B., Sahar, H. K., Kaback, H. R. and Lodish, H. F. (1988) *Cell* **55**, 281-290.

Tominga, M., Komiya, I., Johnson, J. H., Inman, L., Tausif, A., Moltz, J., Crider, B., Stefan, Y., Baetens, D., McCorkle, K., Orci, L. and Unger, R. H. (1986) *Proc. Natl. Acad.Sci. U.S.A.* **83**, 9749-9753.

Trimble, N. S., Linial, M. and Scheller, R. H. (1991) *Annu. Rev. Neurosci.* **14**, 93-122.

Turner, R. J. and Moran, A. (1982) *Am. J. Physiol.* **242**, F406-F414.

Vannucci, S. J., Nishimura, H., Satoh, S. Cushman, S. W., Holman, G. D. and Simpson, I.A. (1992) *Biochem. J.* **288**, 325-330.

Vischer, U., Blondel, B., Wollheim, C. B., Hoppner, E., Seitz, H. J. and Lynedjian, P. B.(1987) *Biochem. J.* **241**, 249-255.

Waddell, I., Scott, H., Grant, A. and Burchell, A. (1991) *Biochem. J.* **275**, 3633-369.

Waddell, I. D., Zomerschoe, A. G., Voice, M. W. and Burchell, A. (1992) *Biochem. J.* **286**, 173-177.

Watanabe, T., Smith, M. M., Robinson, F. W. and Kono, T. (1984) *J. Biol. Chem.* **259**, 13117-13122.

Werner, H., Adamao, M., Lowe, J. L. J., Roberts, C. T. J. and LeRoith, D. (1989) *Mol. Endocrin.* **3**, 273-279.

Wheeler, T. J., Hinkle, P. C. (1985) *Annu. Rev. Physiol.* **47**, 503-517.

Weinhouse, S. (1976) *Curr. Top. Cell. Reg.* **11**, 1-50.

White, M. K., Rall, T. B. and Weber, M. J. (1991) *Mol. Cell. Biol.* **11**, 4448-4454.

White, M. K. and Weber, M. J. (1989) *Nature* **340**, 103-104.

Whitesell, R. R. and Abumrad, N. A. (1986) *J. Biol. Chem.* **261**,15090-15106.

Widmaier, E. (1992) *Horm. Met. Res.* **24**, 147-153.

Widmaier, E. P., Plotsky, P. M., Sutton, S. W. and Vale, W. W. (1988) *Am. J. Physiol.* **255**, E757-E763.

Williams, T. F., Exton, J. H., Park, C. R. and Regen, D. M. (1968) *Am. J. Physiol.* **215**, 1200-1209.

Yang, J., Clark, A. E., Harrison, R., Kozka, I. J. and Holman, G. D. (1992) *Biochem. J.* **281**, 809-817.

Yang, J. and Holman, G. D. (1993) *J. Biol. Chem.* **268**, 4600-4603.

Yano, H., Seino, Y., Inagaki, N., Hinokio, Y., Yamamoto, T., Yasuda, K., Masuda, K., Someya, Y. and Imura, H. (1991) *Biochem. Biophys. Res. Commun.* **174**, 470-477.

Zorzano, A., Wilkinson, W., Kotlair, N., Thoidis, G., Widzinoski, B. E., Ruoko, A. E. and Pilch, P. F. (1989) *J. Biol. Chem.* **264**, 12358-12363.