STUDIES OF THE EXOCYTIC SNARES INVOLVED IN GLUT4 TRANSLOCATION

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By

Fiona Marie Brandie

Division of Biochemistry & Molecular Biology
Institute of Biomedical & Life Sciences
University of Glasgow
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Abstract

A specialised example of vesicular traffic is the translocation of the glucose transporter Glut4 from intracellular storage compartments to the plasma membrane of fat and muscle cells in response to insulin. In the basal state the majority of Glut4 is held intracellularly, sequestered away from the constitutively recycling endosomal pathway, in a proposed population of specialised Glut4 Storage Vesicles (GSVs). Insulin stimulates glucose transport into adipose cells by promoting the translocation of these GSVs to the plasma membrane where they fuse increasing the Glut4 levels at the cell surface and therefore significantly increasing facilitative glucose transport.

The ability of insulin to stimulate glucose transport into muscle and adipose tissue, the main sites of glucose uptake, is central to the ability of insulin to regulate whole body glucose homeostasis. In individuals with type 2 diabetes this ability of insulin to stimulate glucose transport is impaired. The incidence of type 2 diabetes is increasing rapidly and therefore understanding the molecular basis of insulin-stimulated glucose uptake is of fundamental importance. It has been over 25 years since the first evidence that insulin stimulation led to a translocation of glucose transport from an intracellular site to the plasma membrane of insulin responsive cells. Over this period of time significant advances have been made in the understanding of insulin-stimulated glucose uptake, both in the field of insulin signalling and Glut4 trafficking, however the intersection between these two processes is yet to be established. One major advance in the knowledge of Glut4 trafficking was the identification of the SNARE machinery involved in the fusion of GSVs to the cell surface. In eukaryotes all intracellular trafficking events are facilitated by a family of highly conserved proteins called SNAREs.

GLUT4 containing vesicles are enriched in VAMP2, while the plasma membrane of adipocytes is enriched in syntaxin 4 and SNAP23, which together serve as a t-SNARE complex. *In vitro* these three SNAREs form a highly stable core complex and several studies show that these three proteins mediate the fusion of Glut4-containing vesicles with the plasma membrane. Whether this fusion event is regulated by insulin is yet to be established.

The fusion process facilitated by SNARE proteins has been successfully reconstituted *in vitro* using recombinant proteins expressed in *E. coli*. Using the exocytic neuronal

SNAREs in this assay it was demonstrated that SNAREpins, that is the complex formed between cognate sets of v- and t-SNAREs, are necessary and sufficient to fuse artificial membranes. In Chapter 3 as the first step towards studying the regulation of fusion facilitated by syntaxin 4, SNAP23 and VAMP2, an *in vitro* fusion assay using these SNAREs was established. The three SNARE proteins were successfully expressed and purified from *E.coli* prior to reconstitution into synthetic liposomes that were subsequently analysed for fusion. The results of this assay show that these SNARE proteins in isolation are capable of fusing artificial membranes, a fact previously assumed but never definitively shown.

In vivo membrane fusion is believed to be controlled by a number of different proteins and lipids species. It has been demonstrated that the t-SNAREs involved in Glut4 exocytosis are localised to lipid raft domains, isolated as detergent resistant membranes (DRMs), in the mouse adipocyte line 3T3-L1. Whether targeting of these proteins to lipid raft domains plays a role in regulating the fusion SNAREs facilitate remains to be established. To investigate whether the main lipid species found in detergent resistant membranes play a role in SNARE facilitated membrane fusion cholesterol and sphingomyelin were introduced into the *in vitro* fusion assay established in Chapter 3. Unfortunately due to the vast difference in size of liposomes produced on introduction of these lipids the data could not be fully analysed, however it appears that inclusion of these lipids is inhibitory to fusion. In adipocytes SNAP23 is subjected to palmitoylation on conserved cysteine residues in the linker domain between its two SNARE domains. Recently it has been shown that SNAP25, a homologue of SNAP23, is palmitoylated when purified from baculovirally infected insect cells. In order to purify the t-SNARE complex of syntaxin 4 and SNAP23 from insect cells and determine the palmitoylation state of SNAP23 a baculovirus was constructed. The t-SNARE complex was successfully purified from insect cells and SNAP23 appeared to be palmitoylated within these cells. Due to time limitations it was unfortunately not possible to carry out fusion assays using this t-SNARE complex to investigate whether palmitoylation influences fusion facilitated by these SNAREs.

One family of proteins known to play an essential role in SNARE-mediated membrane fusion is the Sec1/Munc 18 (SM) family. A member of the SM family of proteins Munc 18c has been isolated from adipocytes and this isoform appears to specifically interact with syntaxin 2 and 4. The N terminus of plasma membrane syntaxins is highly conserved and has been shown to form a three-helix bundle, which is capable of binding to the SNARE

domain holding such syntaxins in a "closed" conformation, which is incapable of forming SNARE complexes. In syntaxin 1a, the neuronal homologue of syntaxin 4, the adoption of this conformation has been shown to be essential for interaction with its cognate SM protein Munc 18a as the introduction of mutations which inhibit the adoption of this conformation abolish the binding of this SM protein. In order to investigate whether syntaxin 4 adopts a similar conformation through which it interacts with Munc 18c equivalent mutations were introduced into the recombinant cytoplasmic domain and changes in conformation were investigated using circular dichroism and limited proteolysis. These experiments indicated that the introduction of these mutations led to a more "open" conformation and binding experiments with recombinant Munc 18c supported monomeric syntaxin 4 existing in a "closed" conformation, as the introduction of these mutations inhibited the interaction between these two proteins. This study has highlighted novel insights into the interaction of Munc 18c with syntaxin 4.

To Mum, Dad and Lesley,

with all my love,

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Abbreviations

AEBSF	4-(2-Aminoethyl)benzenesulphonyl fluoride
APS	Ammonium persulfate
Bp	Base pairs
CD	Circular dichroism
cDNA	Complementary deoxyribonucleic acid
Da	Dalton
DMSO	Dimethlysulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside (5')-triphosphate
DOPS	1,2-dioleoyl phosphatidylserine
DRM	Detergent resistant membrane
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EM	Electron microscopy
ER	Endoplasmic reticulum
g	Gravitation (10 $m.s^{-2}$)
Glut	Glucose transporter
GST	Glutathione S-transferase
GSV	Glut4 storage vesicle
HEPES	N-2-hydroxyethylpiperazne-N'-2-ethanesulphonic acid
HCl	Hydrochloric acid
HIS-tag	Hexa-histidine sequence
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-D-Thiogalactopyranoside

IRS	Insulin receptor substrate
kb	Kilobase
M	Molar
mA	Milliamps
NBD-DPPE	(N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine
NSF	N-ethylmaleimide sensitive factor
O.D.	Optical density
OG	n-octyl-β-D-glucopyranoside
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
Rhodamine-DPPE	N-(Lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine
rpm	rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf	Spodoptera frugiperda
SNAP	Soluble NSF attachment protein
SNAP25	Synaptosomal protein of 25 kDa

SNARE	SNAP receptor
TAE	Tris-acetate EDTA
TEM	Transmission Electron Microscopy
TEMED	N, N, N', N' -- tetramethylenediamine
TGN	*Trans* Golgi network
Tris	Tris(hydroxymethyl)aminoethane
Tween 20	Polyoxyethylene sorbitan monolaurate
VAMP	Vesicle associated membrane protein
v/v	volume/volume ratio
w/v	weight/volume ratio

Chapter 1

Introduction

1 Introduction

1.1 Insulin-stimulated glucose uptake and diabetes

Although it has been over 25 years since the first evidence that insulin causes translocation of glucose transport activity from an intracellular storage site to the plasma membrane of insulin responsive cells (Cushman and Wardzala, 1980;Suzuki and Kono, 1980) the molecular mechanisms underlying this translocation are yet to be fully elucidated. Over this period of time several advances have been made in the understanding of insulin signalling and Glut4 trafficking. However the intersection between these two processes has so far remained out of reach.

Two tissues within the body, muscle and adipose, play key roles in the maintenance of whole body glucose homeostasis by acting as the main sites of glucose uptake in response to insulin. The insulin-stimulated glucose uptake into these cells is mainly mediated by the glucose transporter Glut4 (Mueckler, 1994). Exposure to insulin causes a rapid and substantial increase in this isoform at the surface of these cells following translocation from an intracellular store that results in the increase in facilitative glucose uptake (Slot *et al.*, 1991a;Slot *et al.*, 1991b). Defects in this system lead to a condition known as diabetes. There are two main types of diabetes; type 1 which is principally caused by the autoimmune destruction of the pancreatic β-cells that secrete insulin and type 2 which is due to insulin resistance in the peripheral tissues (Saltiel, 2001).

Insulin resistance, which is the hallmark of type 2 diabetes, is defined as a decrease in the ability of insulin to stimulate this translocation of Glut4 and thus glucose uptake. Potential defects leading to the impairment of translocation include deficient signalling upstream of the translocation step, abnormalities within the trafficking machinery or indeed mistargetting of Glut4 within the cells. Due to this decreased ability to clear postprandial glucose hyperglycaemia results and this has been associated with many serious health complications (Zimmet *et al.*, 2001). Since insulin resistance is on the increase in the western world, with an estimated 220 million people suffering from diabetes by 2010, (Zimmet *et al.*, 2001) defining the events that lead to the translocation and insertion of Glut4 into the plasma membrane is of particular interest. The trafficking of this glucose transporter involves a number of fundamental processes that must occur in every cell to maintain the functional identity of membrane bound organelles.

1.2 Glucose transporters

A family of highly conserved and highly related transporters called Gluts, short for Glucose transporters, carries out facilitative glucose transport in the body. Thirteen mammalian glucose transporters, each derived from a separate gene, have been identified thus far and each family member is predicted to be composed of 12 membrane-spanning domains with the N- and C-termini of the protein orientated into the cytosol as depicted in Figure 1.1. Glut family members vary in their tissue distribution. Of these thirteen proteins only four (Glut1, Glut2, Glut3 and Glut4) have thus far been shown to be authentic glucose transporters (Gould *et al.*, 1991).

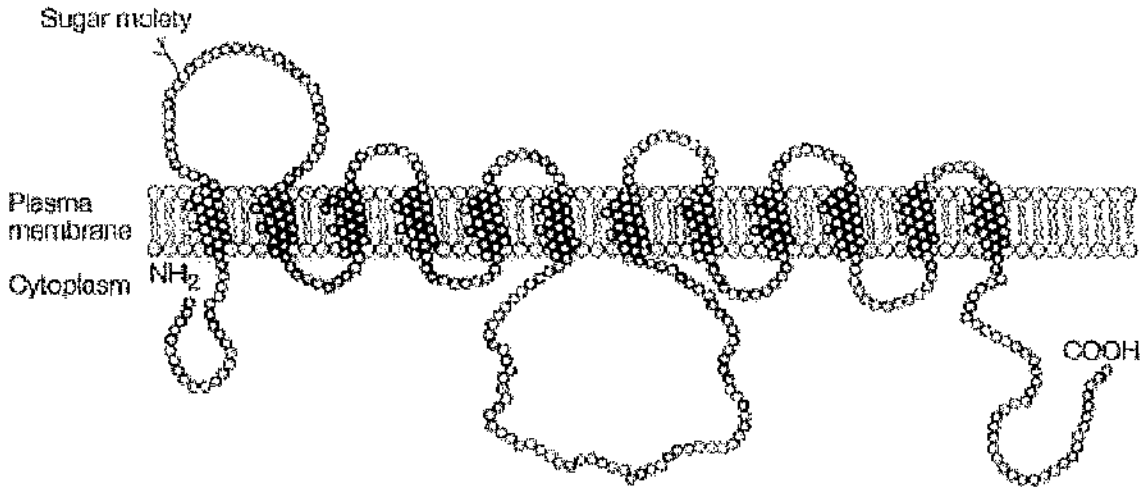


Figure 1.1: The overall structure of the Glucose transporters

The glucose transporters are proposed to have 12 membrane spanning domains with the N- and C-termini of the protein being orientated into the cytosol. Helices 1 and 2 are connected by a large extracellular loop, while helices 6 and 7 are connected by a large intracellular loop. (Taken from Bryant *et al.*, 2002)

In order to avoid damage due to hyper- or hypoglycaemia the body must tightly regulate whole body glucose homeostasis. Glut4 is highly expressed in adipose and striated muscle however, these tissues also express low levels of Glut1 (Mueckler, 1994). The subcellular distribution of each of these transporters suggests they play distinct roles in the maintenance of whole body glucose homeostasis. Glut1 is considered to be the 'housekeeping' isoform since it is equally distributed between intracellular compartments and the plasma membrane under basal conditions and its presence at the cell surface is only slightly increased in response to insulin (Calderhead *et al.*, 1990). In contrast to Glut1, Glut4 is extensively recruited to the plasma membrane in response to insulin and is almost completely excluded under basal conditions (Slot *et al.*, 1991a;Slot *et al.*, 1991b). Glut4 is therefore considered to be the insulin responsive glucose transporter.

1.2.1 Glut4

In 1980 two independent investigations into insulin action on adipose cells demonstrated that insulin caused the movement of glucose transport activity (later cloned (Garcia and Birnbaum, 1989;Charron *et al.*, 1989;Kaestner *et al.*, 1989;James *et al.*, 1989) and called Glut4) from an intracellular location to the plasma membrane (Cushman and Wardzala, 1980;Suzuki and Kono, 1980). This was subsequently shown to also occur in the other insulin responsive tissues, namely heart (Watanabe *et al.*, 1984) and skeletal muscle (Hirshman *et al.*, 1990). Insulin was later shown to cause this movement of Glut4 by significantly increasing the rate of exocytosis of the transporter while only slightly decreasing the rate of internalisation of Glut4 by endocytosis (Satoh *et al.*, 1993), leading to an overall increase in surface Glut4 and therefore facilitative glucose transport. The majority of subsequent research on Glut4 and its trafficking has been undertaken in adipocytes.

1.3 Membrane Trafficking

The inside and the outside of a cell are clearly defined by a boundary consisting of biological membrane termed the plasma membrane. As well as making up the boundary of the cell, biological membranes also define the intracellular boundaries between the different organelles of the cell. Biological membranes are extremely dynamic, and the transport of their constituent lipids and proteins must be highly organised and controlled in

order to maintain cellular integrity. There are two main vesicular trafficking pathways in the cell, the secretory pathway and the endocytic pathway.

1.3.1 Secretory Pathway

The secretory pathway comprises several morphologically distinct compartments through which proteins must successfully traffic on their way to the cell surface. During this trafficking process proteins are folded, quality controlled and post-translationally modified. Proteins to be secreted are initially synthesised in the endoplasmic reticulum (ER), they are then moved to, and through, the Golgi apparatus, where they are processed and sorted, before being packaged into secretory vesicles in which they are transported to the surface of the cell prior to exocytosis and secretion (reviewed in van Vliet *et al.*, 2003).

The secretory pathway terminates with the fusion of secretory vesicles to the plasma membrane, which is termed exocytosis (Jahn, 2004). Exocytosis serves two functions: the first to integrate the lipids and incorporated membrane proteins into the plasma membrane and secondly to release the contents of the secretory vesicle to the extracellular space. Exocytosis can occur in two distinct ways. It can occur constitutively, in all cells, where secretory vesicles are continuously fusing with the plasma membrane in a stimulus-free manner. In specialised secretory cells, such as neurons and pancreatic beta cells, regulated exocytosis can also occur where stored secretory vesicles only fuse to the plasma membrane following a specific stimuli. The insulin-stimulated exocytosis of vesicles enriched in Glut4 in insulin-responsive cells is an example of regulated exocytosis.

1.3.2 Endocytosis

Endocytosis is the mechanism the cell utilises to internalise membrane and molecules from its surface (reviewed in Maxfield and McGraw, 2004). Following endocytosis molecules are transported to the endosomes of the cell where they are sorted and packaged for further transport. The rate of endocytosis from the cell surface is thought to be regulated. In insulin response cells insulin stimulation is thought to lead to a decrease in endocytosis of Glut4, that normally occurs in the basal state to recycle cell surface Glut4, which along with the substantial increase in exocytosis causes an increase in cell surface Glut4 levels (Satoh *et al.*, 1993).

1.3.3 *Trafficking of Glut4*

Several studies have focused on the intracellular location of Glut4 and how insulin stimulates the movement of this transporter to the cell surface. It is estimated that in the absence of insulin the majority of Glut4, as much as 95 %, is held within the cell (Smith *et al.*, 1991). Using immunoelectron microscopy of cryosections of white and brown adipose tissue in the basal state, Glut4 was localised to several intracellular locations including the endosomes and the *trans*-Golgi network (TGN) (Slot *et al.*, 1991b). However, the majority of Glut4 (approximately 80 %) was shown to be associated with a population of small vesicles and tubules which are distinct from the TGN but which cluster around the TGN, the early or late endosome and close to the plasma membrane (Slot *et al.*, 1991b). Upon insulin stimulation there was a distinct movement of Glut4 from these intracellular compartments to the plasma membrane of the cell resulting in a surface increase of Glut4 in the region of 40-fold (Slot *et al.*, 1991b). The same shift from intracellular tubulo-vesicular elements to the plasma membrane was also observed in cardiac myocytes and muscle (Slot *et al.*, 1991a;Ploug *et al.*, 1998).

In 1996 work using 3T3-L1 adipocytes and endosomal ablation (Livingstone *et al.*,1996) suggested the existence of two separate pools of intracellular Glut4: one associated with endosomes and the other distinct from the recycling system which was insulin sensitive (Livingstone *et al.*, 1996). The idea of a separate insulin responsive pool of Glut4 has been supported by a study on skeletal muscle which suggested the presence of a highly insulin responsive population of Glut4-containing vesicles (Aledo *et al.*, 1997) and subcellular fractionation of 3T3-L1 adipocytes (Hashiramoto and James, 2000). These specialised vesicles are now referred to as Glut4 storage vesicles (GSVs) and have been estimated to contain approximately 60% of the total cellular Glut4 (Livingstone *et al.*, 1996).

The simplest model of Glut4 trafficking would suggest that Glut4 is held in a static population of GSVs awaiting release in response to insulin. However, Glut4 is found in the endosomes and TGN in the absence of insulin (Slot *et al.*, 1991a;Slot *et al.*, 1991b). Specialised GSVs have been suggested to traffic in two cycles with GSVs trafficking between both the cell surface and the endosomes in one cycle (cycle 1) and between the TGN and endosomes in the other cycle (cycle 2) (Bryant *et al.*, 2002) as shown in Figure 1.2. The trafficking of this isoform between the TGN and endosomes is proposed to account for the relatively low levels of Glut4 observed at the cell surface and the presence

of this isoform in the TGN in the basal state. In response to insulin it is proposed that Glut4 containing vesicles trafficking in this second cycle are released and allowed to fuse to the plasma membrane (Bryant *et al.*, 2002).

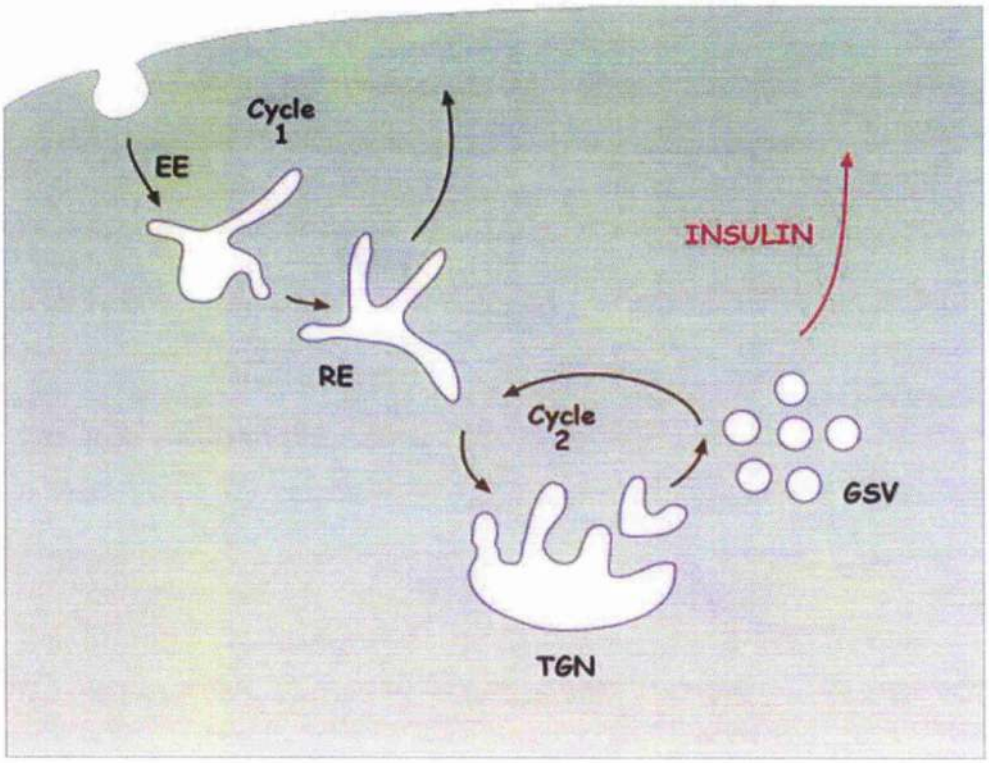


Figure 1.2: The proposed cycles of Glut4 trafficking within the cell

Glut4 has been proposed to cycle in two separate intracellular cycles. In the first cycle Glut4 recycles between the plasma membrane, early endosome (EE) and recycling endosomes (RE). In the second cycle Glut4 is held in a specialised GSV compartment, which recycle between the TGN and RE in the basal state. Upon insulin stimulation GSVs, in cycle 2, are released from retention within the cell and are translocated to the plasma membrane where they fuse and allow for the rapid and substantial increase in facilitative glucose transport.

1.4 The regulation of Glut4 trafficking by Insulin

The discovery of an insulin-responsive Glut isoform prompted research into how insulin stimulates the mobilisation of the intracellular store of Glut4 to the plasma membrane. Insulin signalling is initiated by the binding of insulin to the insulin receptor present on the surface of insulin responsive cells. The insulin receptor, a member of the family of tyrosine kinase receptors, exists as a preformed heterotetramer consisting of 2 α and 2 β subunits linked by disulfide bonds. The binding of insulin to the extracellular α subunits leads to conformational change of the receptor, which enables the transphosphorylation of tyrosine residues present on the β subunits (reviewed in Holman and Kasuga, 1997). This results in a number of docking sites for molecules, that contain SH2 (Src-homology-2) domain and phosphotyrosine-binding (PTB) domains, which are involved in downstream insulin signalling events. The activated insulin receptor recruits amongst other proteins members of the insulin receptor substrate (IRS) family. For a long time one downstream pathway involving the recruitment and activation of IRS proteins, which in turn recruit and activate phosphatidylinositol 3-kinase (PI3K), was thought to be adequate to stimulate Glut4 translocation to the surface of insulin responsive cells. However, recently this idea has been challenged by the discovery of another signalling pathway, which seems to localise to specific domains in the plasma membrane.

1.4.1 PI3K dependent pathway

The 'classical' insulin-signalling cascade involves the activation of insulin receptor substrate (IRS) by phosphorylation following its recruitment to the activated insulin receptor (reviewed in Khan and Pessin, 2002). There are four isoforms of IRS, -1, -2, -3 and –4 (Sun *et al.*, 1991;Sun *et al.*, 1995;Sciacchitano and Taylor, 1997;Fantin *et al.*, 1999). Only IRS-1, -2 and –3 are expressed in adipocytes from rat and mice (Giovannone *et al.*, 2000). Several studies suggest that the IRS proteins play complimentary roles however, in adipocytes IRS-1 is thought to be the main target of insulin (Quon *et al.*, 1994;Kaburagi *et al.*, 1997). Following tyrosine phosphorylation by the activated insulin receptor IRS can act as a docking protein for effectors containing SH2 domains.

The phosphorylation of IRS proteins by the activated insulin receptor leads to the recruitment and activation of the p85/p110-type PI3K (reviewed in Khan and Pessin, 2002). This recruitment brings the PI3K into close proximity with its substrate,

phosphatidylinositols, located in the plasma membrane. Studies have demonstrated that the activation of PI3K is necessary for insulin stimulated glucose uptake (Kotani *et al.*, 1995). Once activated PI3K catalyses the phosphorylation of the D-3 position of inositol rings and therefore leads to, among other reactions, the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 recruits to the plasma membrane and activates phosphoinositide-dependent kinase 1 which then goes on to phosphorylate and activate Protein Kinase B (PKB) (Alessi *et al.*, 1997) and the atypical Protein Kinase C (PKC) isoforms PKCλ and PKCζ (reviewed in Watson and Pessin, 2001).

Following the discovery of this 'classical' pathway evidence emerged for the requirement of another PI3K-independent insulin signalling pathway for Glut4 translocation. While β1-integrin stimulated the activation of PI3K and PKB it had no effect on Glut4 translocation in adipocytes (Guilherme and Czech, 1998). Platelet-derived growth factor and interleukin 4 were also shown to be able to activate PI3K but were unable to stimulate Glut4 translocation or glucose transport in 3T3-L1 adipocytes and L6 muscle cells respectively (Isakoff *et al.*, 1995). The existence of an alternative pathway was further supported when it was observed that the introduction of membrane permeant esters of PI3P, thought to be sufficient to activate the downstream events from PI3K, into adipocytes were unable to stimulate glucose uptake (Jiang *et al.*, 1998). In addition manipulation of the interaction between the insulin receptor and the insulin receptor substrate also had no effect on glucose transport in adipocytes (Sharma *et al.*, 1997). These observations led to a hunt for other pathways that could be responsible for the increase in glucose transport observed in response to insulin.

1.4.2 PI3K independent pathway

A second, PI3K-independent, pathway was identified by Baumann and colleagues (Baumann *et al.*, 2000). In this pathway the activated insulin receptor is proposed to recruit Cbl, a homologue of the transforming v-Cbl oncogene, through its interaction with two adaptor proteins APS (Adaptor protein with a pleckstrin homology and an Src homology 2 domain) (Liu *et al.*, 2002) and CAP (Cbl-associated protein) (Ribon *et al.*, 1998;Kimura *et al.*, 2001). This association promotes the tyrosine phosphorylation of Cbl, which facilitates the translocation of this complex to specialised membrane domains in the plasma membrane termed lipid rafts (which will be discussed later). The association of

this complex with lipid rafts is thought to be due to the interaction between the lipid raft resident protein flotilin and the sorbin homology domain of CAP (Baumann *et al.*, 2000;Kimura *et al.*, 2001). Following phosphorylation and recruitment to lipid raft domains Cbl recruits the small adaptor protein Crk II that in turn recruits C3G, a guanylnucleotide exchange factor (Chiang *et al.*, 2001). Once recruited, C3G is brought into close proximity with TC10, a member of the Rho family of GTPases (Chiang *et al.*, 2001). C3G activates TC10, by promoting the exchange of GTP for GDP, which is then believed to signal through an as yet uncharacterised mechanism for Glut4 translocation to the plasma membrane (Chiang *et al.*, 2001). There is strong evidence that the lipid raft localisation of this pathway is vital for signalling via this PI3K-independent route (Watson *et al.*, 2001;Kimura *et al.*, 2001).

However, the physiological significance of this second pathway has recently been called into question. SiRNA of many of the key components in the pathway was found to have no effect on insulin stimulated glucose uptake (Mitra *et al.*, 2004), while adipocytes from Cbl knockout mice were not found to be defective in insulin stimulated glucose uptake and muscle from these animals was actually found to have a significant increase in insulin stimulated glucose uptake (Molero *et al.*, 2004).

The intersection between these insulin signalling pathways and the regulatory step of Glut4 trafficking is yet to be established. One possible intersection is at the level of regulation of membrane fusion of vesicles containing Glut4 to the plasma membrane. Membrane fusion takes place through a highly conserved and tightly regulated mechanism.

1.5 Fusion

1.5.1 Tethering, docking and priming

The transport of vesicles, and the delivery of these vesicles to the correct organelle within the cell is of great importance in the maintenance of the integrity of the cell. A large number of proteins, and protein complexes, work together in order to tightly regulate this process. The transport of vesicles within the cell occurs through 4 defined stages, firstly budding of the transport vesicle from the donor membrane, then transport through the cell, which is followed by tethering/docking of the vesicle to the appropriate target membrane and finally membrane fusion. During tethering the transport vesicle is held at a distance

(thought to be greater than 25 nm) from the target membrane through interactions involving tethering proteins or protein complexes, which interact with both the vesicle and target compartment (Pfeffer, 1999). During docking the vesicle is more tightly attached and may be only 5-10 nm from the target membrane (Pfeffer, 1999). During the final membrane fusion event the two membranes integrate into one another through an as yet undefined mechanism.

1.5.2 Membrane Fusion intermediates

Membranes do not fuse spontaneously since the repulsive energy between two opposing membranes is thought to be extremely high. The actual process of how two membranes fuse into one another is still incompletely understood. Two models have emerged as possible paths of membrane fusion. The first involves the formation of a fusion pore, which allows for direct fusion of the two opposing membranes (Chernomordik and Kozlov, 2005). The second involves the formation of a hemifusion intermediate where the proximal leaflets of the two membranes merge, followed by the formation of a fusion stalk which then forms a fusion pore, with the expansion of this pore completing the process of membrane fusion (Chernomordik and Kozlov, 2005). Although there is some debate as to which path membranes fuse through, a family of proteins called SNAREs is known to be essential for this process.

1.5.3 SNAREs

Every eukaryotic membrane fusion event is now known to involve a family of proteins called soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs). SNAREs act as receptors for the cytoplasmic proteins NSF (N- ethylmaleimide sensitive factor) and SNAP (soluble NSF attachment protein), which were known to play a role in membrane trafficking prior to the discovery of SNARE proteins.

SNAREs were biochemically isolated through the purification of bovine brain detergent extract using an affinity column consisting of recombinant NSF and SNAP by Rothman and colleagues in 1993 (Sollner *et al.*, 1993). One of these SNARE proteins had previously been localised to the plasma membrane of presynaptic neurons (Bennett *et al.*, 1992) while another had been localised to small synaptic vesicles (Trimble *et al.*, 1988). The localisations of these SNARE proteins provided the possibility that proteins on

different membranes could interact to facilitate fusion. This discovery led to the formulation of the SNARE hypothesis. The SNARE hypothesis suggests that for every vesicle trafficking event there is a specific v-SNARE on the vesicle and a specific t-SNARE on the target membrane, which interact to achieve the specificity of targeting (Sollner *et al.*, 1993). Thus, using the NSF/SNAP column, Rothman and colleagues purified the prototypes of v and t-SNAREs.

The neuronal SNARE proteins identified by this study were the v-SNARE VAMP, a member of the synaptobrevin family, and the t-SNAREs SNAP25 (synaptosomal protein of 25 kDa) and syntaxin 1A. Many homologues of these proteins have since been discovered. The family of SNARE proteins are now characterised by a homologous domain of ~60 amino acids known as the SNARE motif through which they interact with each other to facilitate membrane fusion.

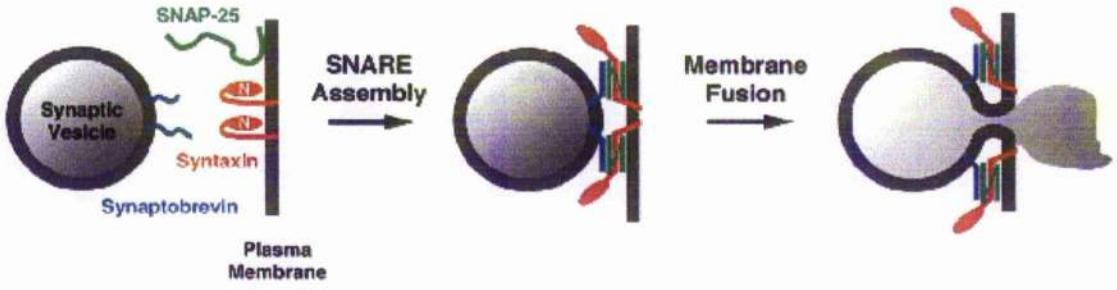


Figure 1.3: Model of SNARE action

Following the docking of the vesicle (in this case a synaptic vesicle) to the target membrane (in this case the plasma membrane) SNAREs, one set on the vesicle and one set on the target membrane, come together to form a *trans*-SNARE complex known as the SNAREpin. *In vitro* the formation of this SNAREpin has been shown to be necessary and sufficient for membrane fusion. It is thought that the energy from the zippering of the SNAREpin may be capable of overcoming the repulsion between the two membranes and thus facilitate fusion. Following fusion the v- (synaptobrevin in this diagram) and t- SNAREs (Syntaxin and SNAP-25 in this diagram) are said to be in a *cis*-SNARE complex with both their transmembrane domains in the same membrane. (taken from Barrick and Hughson, 2002).

1.5.3.1 Classification of SNAREs

Originally the SNAREs were classified using their intracellular location with v-SNAREs being vesicle bound while t-SNAREs were found on target membranes. However, the discovery that v- and t-SNAREs could coexist on the same membrane led to their reclassification into Q- or R-SNAREs on a structural basis. The Q- and R- classification is based on the amino acid residue present at a highly conserved position within their SNARE domain, which is involved in the formation of the central layer of the complex (Fasshauer *et al.*, 1998). For R-SNAREs (e.g. synaptobrevin) this residue is an arginine while for Q-SNAREs (e.g. syntaxin 1A and SNAP25) the residue is a glutamine (Fasshauer *et al.*, 1998). The t-SNARE family has been further classified into Qa, which represents the syntaxins, and Qb, and Qc referring to the N and C SNARE motifs of SNAP25 homologues respectively.

1.5.3.2 R-SNAREs (VAMPs)

R-SNAREs, also known as VAMPs (vesicle associated membrane protein) or synaptobrevins, were first identified as components of synaptic vesicles prior to their identification as SNAP-binding proteins (Trimble *et al.*, 1988). Synaptobrevin family members are small conserved proteins of ~120 amino acids. Ten mammalian R-SNAREs have been identified thus far (Steegmaier *et al.*, 1999). Although all known R-SNAREs have predicted transmembrane domains which are thought to anchor them to the membrane three R-SNARE homologues, VAMP2, snc-1 and snc-2 have recently been demonstrated to be palmitoylated on a conserved cysteine residue (Couve *et al.*, 1995;Veit *et al.*, 2000). Whether this palmitoylation of the R-SNAREs has any physiological significance is yet to be investigated.

1.5.3.3 Syntaxins

Syntaxins, also known as Qa SNAREs, were first identified in neurons (Inoue *et al.*, 1992), prior to their identification as SNAP-binding proteins (Bennett *et al.*, 1992). The syntaxins were first described as two 35 kDa proteins, now known as 1A and 1B, which showed 84 % sequence identity (Bennett *et al.*, 1992). Since then 16 family members have been found expressed in mammalian cells (Teng *et al.*, 2001), making them the largest mammalian SNARE family identified thus far.

All mammalian syntaxins, with the exception of syntaxin 11, are anchored in the membrane by a C-terminal transmembrane tail (Teng *et al.*, 2001). Such proteins are inserted into the membrane post-translationally (Borgese *et al.*, 2003). In addition to the transmembrane domain and a single SNARE domain the syntaxins also have a large N-terminal domain that makes up roughly two thirds of the molecule (Fernandez *et al.*, 1998). This N-terminal domain encodes 3 α-helices (Ha, Hb and Hc), which have been predicted using NMR to form a three-helix bundle (Fernandez *et al.*, 1998). This N-terminal domain is highly conserved in exocytic syntaxins (Fernandez *et al.*, 1998). In some syntaxins this N-terminal domain has been shown to fold back and interact with the SNARE domain holding the protein in a closed conformation however, in other syntaxins this does not appear to occur.

The syntaxin homologues show different levels of expression in different tissues (Bennett *et al.*, 1993). However, their location within different cell types appears to be isoform specific. Syntaxins 1, 2, 3 and 4 have all been found to localise predominantly to the plasma membrane where they mediate constitutive and regulated vesicle transport to the plasma membrane (Teng *et al.*, 2001).

1.5.3.4 SNAP25 family

To date only 4 members of the SNAP25 family have been identified in mammalian cells. While the expression of SNAP25 is restricted to neuronal and neuroendocrine cells, the other members of the family SNAP23, SNAP29 and SNAP47 are ubiquitously expressed (Wang *et al.*, 1997;Steegmaier *et al.*, 1998;Holt *et al.*, 2006). SNAP23 shares roughly 60 % sequence homology with SNAP25 (Hodel, 1998).

The N and C-terminal domains of SNAP25 family members are predicted to form coiled-coil domains that bind syntaxin, separated by a flexible linker region. Unlike other SNAREs, the SNAP25 family does not contain a membrane-spanning domain. SNAP25 and SNAP23 are instead anchored to the membrane through the covalent attachment of palmitate groups to a number of cysteine residues in a conserved cysteine rich domain situated in the linker domain which bridges the two coiled-coil domains or through interactions with other proteins including syntaxin (Vogel and Roche, 1999). SNAP29 lacks this cysteine rich domain and is thought to localise to membranes solely through its interaction with syntaxin or another membrane bound protein (Steegmaier *et al.*, 1998).

1.5.3.5 *Structure of SNARE complexes*

The neuronal SNARE complex, the first SNARE complex identified consisting of syntaxin 1A, SNAP25 and VAMP2, remains the most widely characterised SNARE complex. SNARE proteins come together through their highly conserved SNARE motifs, adjacent to their membrane anchor, to form the SNARE core complex.

The crystal structure of the complex formed by the SNARE domains of the neuronal SNAREs, at 2.4 Å, revealed that the four SNARE motifs of these proteins come together to form a parallel twisted 4 helical bundle (~12 nm in length) with syntaxin 1A and VAMP2 contributing one helix and SNAP25B contributing 2 helices (Sutton *et al.*, 1998). Spin labelling electron paramagnetic resonance spectroscopy of this SNARE core complex has supported a parallel configuration of the helices (Poirier *et al.*, 1998b). The SNARE motif is made up of a heptad pattern of hydrophobic residues and the formation of the SNARE complex leads to these residues residing on the same face of the helix, typical of a coiled coil interaction. The interaction of highly conserved amino acids in each helix forms the interface between the helices (Fasshauer *et al.*, 1998). Three glutamine residues, one from syntaxin 1A and 2 from SNAP25, hydrogen bond with an arginine side chain from VAMP2 present in the central zero ionic layer of the core complex (Fasshauer *et al.*, 1998). The residues that participate in this interaction form the basis of the classification of SNAREs as outlined in section 1.5.3.1. The outer surface of the helical bundle has four grooves (Sutton *et al.*, 1998) that have been proposed to function as binding sites for interacting proteins. Despite limited sequence homology between the synaptic SNARE complex and the endosomal SNARE complex of Syntaxin 7, Vti1b, Syntaxin 8 and VAMP8 the crystal structure derived from the SNARE domains of these SNAREs supports this overall structure suggesting this structure is highly conserved throughout the SNARE family (Antonin *et al.*, 2002).

SNARE complexes appear to be extremely stable structures. The ternary complex formed by syntaxin 1A, SNAP25 and VAMP is resistant to the denaturing detergent SDS (Hayashi *et al.*, 1994) while both the binary complex of syntaxin 1A and SNAP25 as well as the ternary complex formed from the addition of VAMP are protected from protease digestion by trypsin compared to the SNAREs alone (Poirier *et al.*, 1998a). The SNAREs are proposed to zipper up from their N to C terminus to form a parallel complex, which presumably brings the two membranes in which they are anchored into close apposition

(Fasshauer and Margittai, 2004). Although the crystal structures of the SNARE complex revealed a tightly packed parallel 4-helix bundle it was found that the cytoplasmic domains of individual SNARE proteins, with the exception of syntaxin, are mainly unstructured in solution (Fasshauer *et al.*, 1997a;Fasshauer *et al.*, 1997b). However, upon forming binary or ternary complexes there are major conformational changes and the SNARE domains increase significantly in α-helical content (Fasshauer *et al.*, 1997a;Fasshauer *et al.*, 1997b). The large change in structure observed on formation of the core complex and the high stability of this complex suggests that the formation of such a complex is extremely energetically favourable and it is thought that this may provide the energy to overcome the repulsion between two membranes and facilitate fusion.

In vitro it has been demonstrated that the formation of the binary complex between syntaxin and SNAP25 occurs prior to the binding of synaptobrevin to form the ternary complex (Fasshauer and Margittai, 2004). Since syntaxin and SNAP25 are present on the same membrane it seems likely that the formation of a binary complex occurs prior to binding of VAMP *in vivo*. This is supported by recent FRET experiments using labelled SNAP25 and syntaxin introduced into PC12 cells suggesting the formation of the binary complex occurs prior to stimulation of the cell and thus formation of the ternary complex (An and Almers, 2004). However, *in vitro* data suggests that the binary complex formed from the addition of SNAP25 to syntaxin does not yield a 1:1 ratio within the binary complex but rather 2 syntaxin molecules bind SNAP25, with one SNARE motif occupying the binding site of synaptobrevin (Fasshauer *et al.*, 1997b;Zhang *et al.*, 2002) which synaptobrevin displaces to form the ternary complex (Fasshauer *et al.*, 1997b). However, the homologous yeast binary complex of Sso1p (syntaxin homologue) and Sec 9p (SNAP25 homologue) has been shown to have a 1:1 ratio *in vitro* (Rice *et al.*, 1997) suggesting that *in vivo* a 2:1 complex of syntaxin and SNAP25 may not exist.

1.5.3.6 Fusion in vitro

The fusion process facilitated by SNARE proteins has been successfully reconstituted *in vitro* (Weber *et al.*, 1998). In this assay one population of liposomes are reconstituted with the t-SNARE complex while the other population, which contain a quenched mixture of fluorescent lipids, is reconstituted with the v-SNARE. When the two vesicle populations fuse the fluorescent lipids become more diluted within the membrane and fusion can thus be measured as a function of increasing fluorescence. Using this assay it was demonstrated

that SNAREpins, that is the complex formed between cognate sets of v- and t-SNAREs are necessary and sufficient to fuse artificial membranes (Weber *et al.*, 1998). The results from the *in vitro* fusion assay suggest that the formation of the energetically favourable SNARE complex is alone sufficient to overcome the repulsion between two membranes and facilitate fusion. This assay has been used to study the regulation of SNARE complex formation including the control of specificity.

1.5.3.7 Specificity of SNARE mediated membrane fusion

The discovery that there were many homologues of SNAREs and that these occupied distinct locations within the cell suggested the possibility that SNARE proteins themselves offered a level of specificity within the fusion process. The original SNARE hypothesis proposed that the interaction between specific pairs of t- and v-SNAREs offered specificity in complex formation in a simple lock and key manner. However, individual SNARE domains have been shown to pair with the SNARE domain of numerous SNARE complex partners *in vitro* with the capacity to form highly stable core complexes (Yang *et al.*, 1999;Fasshauer *et al.*, 1999;Tsui and Banfield, 2000) suggesting that the interactions between SNARE domains are not selective. This is not particularly surprising when the high conservation of the interacting residues within the SNARE domains is considered. However, these studies were performed using the cytoplasmic domains of SNAREs in solution and may not accurately reflect the *in vitro* situation where the SNAREs are anchored in membranes which have a strong repulsion against each other. However, studies in *Drosophila* and yeast have demonstrated that the interactions between SNARE domains is also promiscuous *in vivo* since the overexpression of members of the v- ant t-SNARE family respectively can compensate for the deletion of other v and t- SNARE members (Gotte and Gallwitz, 1997;Bhattacharya *et al.*, 2002).

Studies utilising the *in vitro* liposome fusion assay, described in section 1.5.3.6, with reconstituted full-length recombinant yeast SNAREs have demonstrated higher levels of specificity. With one exception, only SNARE pairings thought to be physiologically relevant gave substantial fusion using this system (McNew *et al.*, 2000a). However, there is the possibility that the lack of fusion in some of these pairings may be due to the absence of any additional proteins in the assay. A subsequent study using the same assay demonstrated that on removal of the N-terminal domains of the t-SNAREs specificity for the v-SNARE was not lost suggesting that specificity is encoded solely in the SNARE

motif as opposed to the other domains of the SNARE proteins (Paumet *et al.*, 2004). A higher level of specificity between SNARE pairings *in vivo* has also been demonstrated using an independent cracked cell assay in PC12 cells (Scales *et al.*, 2000).

1.5.3.8 Localisation of SNAREs within the cell

Within the cell SNARE members have distinct locations. The targeting of members of the SNARE family to distinct locations within the cell may be a means of regulating the specificity of fusion at particular compartments. The factors that ultimately control the localisation of SNAREs within the cell are still to be fully established. The typical locations of SNAREs within the mammalian cell are depicted in Figure 1.4.

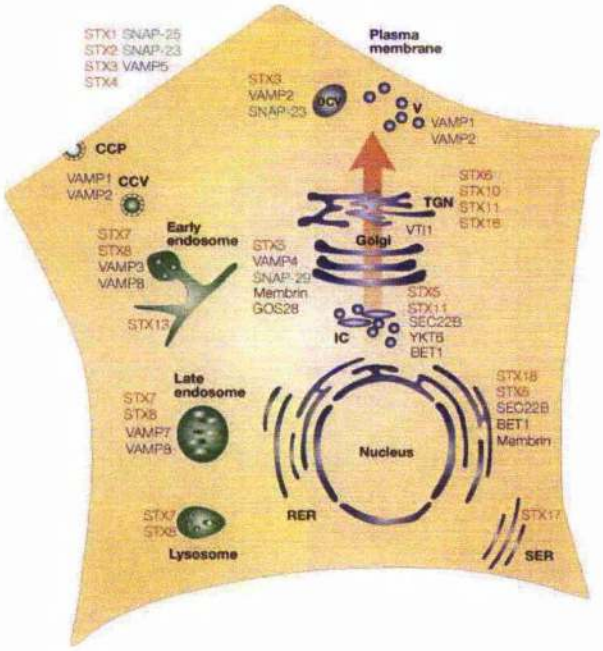


Figure 1.4: The typical location of mammalian SNAREs within the cell

Members of the R- and Q-SNAREs occupy distinct intracellular locations within a typical mammalian cell. (Taken from Chen and Scheller, 2001).

1.5.3.9 Clustering of SNAREs

When viruses fuse with mammalian cells the process is similar to that of intracellular fusion facilitate by SNAREs. However, in the case of viral fusion the protein machinery required for membrane fusion is only present on viral membrane. Membrane fusion involving these viral fusion peptides, which interact with the host plasma membrane to facilitate fusion during viral entry to the cell, has been widely characterised. Electron microscopy and X-ray crystallography studies of the Semliki Forest virus fusion glycoprotein E1 suggests that 5 trimers facilitate the fusion of the virus to the host cell (Gibbons *et al.*, 2004). Kinetic studies on the influenza virus fusion protein hemagglutinin (HA), which facilitates viral fusion to the host endosomal membrane, have suggested that membrane fusion events facilitated by this protein also involve a number of subunits with at least 3 to 4 homotrimers of HA cooperating together to facilitate fusion (Danieli *et al.*, 1996). It is now thought that intracellular membrane fusion in mammalian cells also requires multiple sets of protein subunits, SNARE complexes, however the exact number of SNARE proteins required for each individual exocytic event is presently unknown.

SNARE proteins have been demonstrated to occur in clusters which supports the idea that more than one SNARE complex is need to drive the fusion of one vesicle into the plasma membrane (Sieber *et al.*, 2006). A study of recombinant SNAREs reconstituted into liposomes using AFM revealed that when v- and t-SNAREs interact they form circular arrays (Cho *et al.*, 2002). Using Transmission Electron Microscopy (TEM) native synaptic SNARE complexes, purified from a detergent extract of bovine brain, have been shown to oligomerise into star shaped bundles containing mainly 3 or 4, but also up to 6 complexes (Rickman *et al.*, 2005). Purified recombinant full-length SNAREs also displayed the same pattern of oligomerisation suggesting that the ability to oligomerise is intrinsic to the SNAREs themselves (Rickman *et al.*, 2005). A recent study, which studied the clustering of full-length and a deletion of the N terminus of syntaxin 1a, suggested that the formation of higher order structures of SNARE proteins is primarily mediated by their SNARE domain *in vivo* however these proteins still contained the transmembrane domain (Sieber *et al.*, 2006). However, the ability of purified recombinant SNAREs to form clustered arrays was lost with the deletion of the transmembrane region of syntaxin 1A suggesting that the transmembrane forms the point of oligomerisation (Rickman *et al.*, 2005). Recombinant and native complexes have also been shown to oligomerise into higher order structures using native SDS-PAGE (Rao *et al.*, 2001;Tokumaru *et al.*, 2001). Synaptobrevin and

syntaxin 1 have been shown to homodimerise and heterodimerise through interactions dependent on conserved residues in their transmembrane domains (Laage *et al.*, 2000).

By examining the kinetics of membrane fusion, following addition of increasing amounts of the soluble domain of VAMP2 to a cracked PC12 cell assay, Hua and Scheller estimated that 3 SNARE complexes cooperate in the fusion of one vesicle to the plasma membrane (Hua and Scheller, 2001). Site directed mutagenesis of the syntaxin 1A transmembrane region, amperometry and membrane capacitance measurements have led to a model where 5 to 8 syntaxin transmembrane domains form alpha helices which line a fusion pore and cooperate to facilitate fusion of one vesicle to the target membrane (Han *et al.*, 2004). Controlling the clustering of SNAREs within the cell may be a level of regulation in the fusion process.

1.6 SNAREs involved in Glut4 trafficking

Since the discovery of the neuronal SNARE complex many homologues of the neuronal v- and t-SNAREs have been cloned. Conceptually the recruitment of a pool of intracellular vesicles containing Glut4 is similar to that of neurotransmitter release from neuronal cells. In neuronal cells neurotransmitters are stored in a preformed vesicle population, which upon stimulation fuse with the plasma membrane through a process of synaptic exocytosis, and are retrieved following action by endocytosis (reviewed by Lin and Scheller, 2000) much like Glut4 transporters in insulin responsive cells. These similarities prompted research into the possibility that SNARE proteins were involved in Glut4 storage vesicle traffic.

1.6.1 v-SNAREs

Two members of the synaptobrevin family, VAMP2 and cellubrevin (VAMP3), are expressed in adipocyte cells (Cain *et al.*, 1992;Volchuk *et al.*, 1995). Both of these isoforms colocalise with Glut4 in adipocytes and are enriched on Glut4-containing vesicles (Volchuk *et al.*, 1995). In addition, both of these isoforms showed a similar pattern of insulin-stimulated movement from low density microsomes to the plasma membrane, although to a smaller extent, as Glut4 (Martin *et al.*, 1996). However, when vesicles containing cellubrevin were immunoprecipitated, it was found that although these contained Glut4 they were devoid of VAMP2 (Volchuk *et al.*, 1995), suggesting the

existence of two separate pools of Glut4-containing vesicles. The existence of two pools has been supported by immuno-electron microscopy of rat adipocytes which showed that, although VAMP2 colocalised with Glut4 in a high proportion of intracellular compartments, Glut4 was also present in vesicles which lacked VAMP2 (Ramm *et al.*, 2000). The use of compartmental ablation and subcellular fractionation showed that, while the majority of cellubrevin was found in the endosomal fraction, a large proportion of VAMP2 and Glut4 were found in a separate compartment, suggesting VAMP2 was the isoform more likely to participate in insulin-stimulated Glut4 translocation (Martin *et al.*, 1996).

The discovery of toxins, which specifically cleave individual SNARE proteins, has helped define which sets of SNAREs are involved in particular fusion events. Tetanus toxin light chain, botulinum neurotoxin D and botulinum toxin B have been shown to cleave both VAMP2 and cellubrevin (Cheatham *et al.*, 1996;Macaulay *et al.*, 1997a;Macaulay *et al.*, 1997b;Foran *et al.*, 1999;Randhawa *et al.*, 2000). The introduction of these toxins into adipocytes was shown to inhibit insulin-stimulated translocation of Glut4 to the cell surface (Tamori *et al.*, 1996;Cheatham *et al.*, 1996;Macaulay *et al.*, 1997a;Macaulay *et al.*, 1997b). Although this supported the involvement of a one of the isoforms in insulin stimulated translocation of Glut4 to the plasma membrane, it did not distinguish between the contributions of each isoform. The concomitant introduction of tetanus resistant forms of the two isoforms along with tetanus toxin light chain into L6 myoblasts demonstrated that only VAMP2 was able to rescue the toxin inhibition of insulin stimulated Glut4 translocation (Randhawa *et al.*, 2000). The introduction of recombinant soluble domains of the two isoforms into adipocytes showed that only the soluble domain of VAMP2 was able to substantially inhibit insulin-stimulated Glut4 translocation (Martin *et al.*, 1998;Millar *et al.*, 1999). VAMP2 is thus proposed to regulate the insulin stimulated exocytosis of Glut4-containing vesicles while cellubrevin is proposed to play a role in the constitutive endosomal recycling of the transporter.

1.6.2 t-SNAREs

Adipocytes were found not to express syntaxin 1A or 1B (Volchuk *et al.* 1996;Timmers *et al.*, 1996). Adipocytes do however express syntaxin 2, 3 and 4 (Timmers *et al.*, 1996;Volchuk *et al.*, 1996;Olson *et al.*, 1997). Of the syntaxin isoforms only syntaxin 1A and syntaxin 4 have been shown to bind VAMP2 (Pevsner *et al.*, 1994a). Syntaxin 4 was

shown to be predominantly located at the plasma membrane of adipocytes and its localisation appeared to be unaffected by insulin stimulation (Volchuk *et al.*, 1996;Tellam *et al.*, 1997). The introduction of anti-syntaxin 4 antibodies into adipocytes through permeabilisation or microinjection led to reduced insulin-stimulated glucose transport and Glut4 translocation to the cell surface respectively (Volchuk *et al.*, 1996;Tellam *et al.*, 1997). Introduction of the cytoplasmic domain of syntaxin 4 into adipocytes either through overexpression driven by vaccinia virus or recombinant protein introduced into permeabilised cells also inhibited insulin stimulated Glut4 translocation (Cheatham *et al.*, 1996;Olson *et al.*, 1997). There is therefore strong evidence for syntaxin 4 playing a key role in insulin stimulated Glut 4 translocation.

Using the cytoplasmic domain of syntaxin 4 to screen a cDNA library Ravichandran and colleagues identified a novel SNAP25 isoform, known as SNAP23 or syndet (Ravichandran *et al.*, 1996). This isoform was found to be expressed in numerous tissues (Wang *et al.*, 1997;Wong *et al.*, 1997). SNAP23 was highly expressed in adipocytes where it was mainly localised to the plasma membrane (Wang *et al.*, 1997). Much like syntaxin 4 the localisation of SNAP23 was unaffected by insulin stimulation (Wang *et al.*, 1997;St Denis *et al.*, 1999). The interaction between SNAP23 and syntaxin 4 was confirmed by co-immunoprecipitation from solubilised rat adipocyte plasma membrane and this interaction seemed to be unaffected by insulin stimulation (St Denis *et al.*, 1999). The introduction of antibodies directed against SNAP23 through permeabilisation or microinjection into adipocytes reduced insulin stimulated Glut4 translocation (Rea *et al.*, 1998;Foster *et al.*, 1999). This reduction in insulin-stimulated Glut4 translocation was also observed following the introduction of a SNAP23 peptide encoding the first 24 amino acids into permeabilised adipocytes (Rea *et al.*, 1998). The overexpression of a mutant of SNAP23 able to bind syntaxin 4 but not VAMP2 in adipocytes through the use of adenovirus gave a similar reduction (Kawanishi *et al.*, 2000).

Using surface plasmon resonance Rea and colleagues demonstrated that VAMP2, SNAP23 and syntaxin 4 form an extremely stable SDS-resistant SNARE complex *in vitro* through their SNARE domains as outlined in Figure 1.5A and 1.5B(Rea *et al.*, 1998).

All of these studies strongly suggest that the v-SNARE present on insulin responsive Glut4 storage vesicles VAMP2 interacts with the binary complex formed by syntaxin 4 and SNAP23 present on the plasma membrane to facilitate the exocytosis of the GSV.

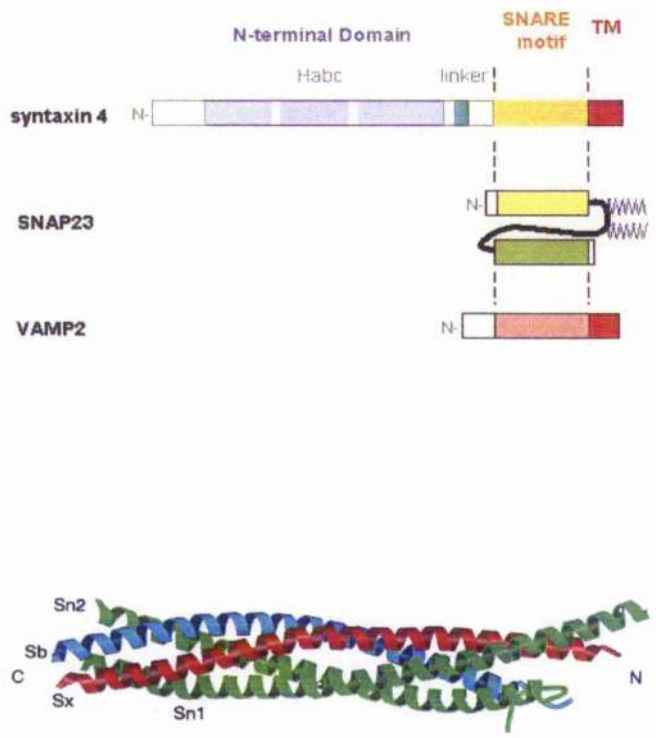


Figure 1.5: The structures of the t- and v- SNAREs involved in Glut4 translocation to the plasma membrane

(A) Plasma membrane syntaxins, including syntaxin 4, contain a C-terminal transmembrane region (red), a C-terminal conserved region termed the SNARE domain (yellow), involved in forming the core complex, which is joined through a flexible linker domain (turquoise) to the N-terminal domain composed of three helices termed Ha, Hb and Hc, collectively known as the Habc domain (blue). SNAP23 is composed of two SNARE domains (yellow and green) connected by a linker that is post-translationally modified by addition of palmitate groups to conserved central cysteine residues. VAMP2 has a short N-terminal region, one SNARE domain (pink) and a C-terminal transmembrane domain through which it is anchored to the membrane (red). (Taken from Grusovin and Macaulay, 2003) **(B) It has been shown that syntaxin 4, SNAP23 and VAMP2 come together through their SNARE domains to form a highly stable SNARE core complex thought to be similar to the structure formed by the neuronal exocytic SNAREs as shown here. Sb = SNARE domain of synaptobrevin (VAMP2, Blue), Sx = SNARE domain of syntaxin 1a (Red), Sn1 and Sn2 = SNARE domains of SNAP25 (green).** (Taken from Sutton *et al.*, 1998)

1.6.3 The exocyst

Following the characterisation of the SNAREs involved in Glut4 translocation to the plasma membrane, the complex that tethers the Glut4 containing vesicle to the plasma membrane prior to fusion was identified.

The exocyst is a large multi-protein complex that tethers vesicles destined for exocytosis with the plasma membrane. The exocyst was initially identified in *Saccharomyces cerevisiae* where it was localised to sites of active exocytosis (TerBush and Novick, 1995;Guo *et al.*, 1999b). The yeast exocyst complex is composed of 8 subunits Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (TerBush *et al.*, 1996;Guo *et al.*, 1999a). Mammalian homologues of all these subunits have since been identified and these are highly conserved (Hsu *et al.*, 1996;Grindstaff *et al.*, 1998;Hsu *et al.*, 1999;Lipschutz *et al.*, 2000;Matern *et al.*, 2001;Ycaman *et al.*, 2001).

Two independent studies have now proposed a role for the exocyst in Glut4 traffic to the plasma membrane. Exo70, a component of the mammalian exocyst complex, was recently found to bind TC10, a component of the insulin-signalling cascade localised to rafts, in a GTP-dependent manner (Inoue *et al.*, 2003). The activation of TC10 is thought to direct the relocation of Exo70 to the plasma membrane (Inoue *et al.*, 2003). Exo70 overexpression significantly increased while the dominant negative version significantly decreased insulin stimulated glucose transport giving strong evidence that Exo70 is required for insulin stimulated glucose uptake (Inoue *et al.*, 2003). In contrast to their effects on glucose transport these two mutants had no effect on the translocation of GFP-Glut4, which may be inserted in the plasma membrane or held under the membrane in a docked vesicle, to the cell surface following insulin stimulation (Inoue *et al.*, 2003). These data strongly suggests that Exo70 must be acting at the site of tethering and docking of Glut4 containing vesicles to the plasma membrane. Consistent with a role in tethering/docking, WT Exo70 increased the insertion of Glut4, monitored using an HA epitope on an exofacial loop of the protein, following insulin stimulation, while the dominant negative mutant inhibited this insertion (Inoue *et al.*, 2003). A role for the exocyst in Glut4 vesicle trafficking was further supported by a second study which utilised adenoviral overexpression of two other subunits of the mammalian exocyst complex, rsec6 and rsec8, leading to an increase in insulin-stimulated glucose transport in 3T3-L1 adipocytes (Ewart *et al.*, 2005).

Sec15p, a yeast exocyst component, has been shown to bind activated Rab GTPase Sec4p, that is normally present on secretory vesicles (Guo *et al.*, 1999b). The interaction between Sec15p and Sec4p is proposed to allow the tethering factor to link the secretory vesicle to its target membrane (Guo *et al.*, 1999b). Whether the mammalian exocyst subunit binds to GTPase Rab4, which has been found to be present on Glut4-containing vesicles, remains to be established.

1.7 Regulation of Fusion

While it is widely accepted that SNAREs are central to the process of membrane fusion within the cell the control of such a fusion step is far from understood. A large number of proteins have been found to interact with the neuronal SNAREs and most of these proteins have conserved homologues in other cell types. These proteins have a variety of effects on SNARE complex assembly; some positive, some negative and in some controversial cases both.

1.7.1 NSF and SNAP

SNARE proteins were originally identified by the ability of the assembled synaptic SNARE complex comprised of syntaxin 1A, SNAP25 and VAMP2 to bind to NSF and SNAP to form a 20 S complex (Sollner *et al.*, 1993). NSF was originally identified as a protein that is inactivated by N-ethylmaleimide and was initially believed to be involved in directly activating membrane fusion (Malhotra *et al.*, 1988). It is now known to be involved in priming SNAREs for fusion by disassembling cis-SNARE complexes in concert with αSNAP (Mayer *et al.*, 1996). Cis-SNARE complexes are formed following a fusion reaction when the v- and t-SNARE reside on the same membrane. The disassembly of such complexes by NSF and SNAP allows for the recycling and reactivation of the SNAREs for further rounds of fusion. NSF and αSNAP are both expressed in rat adipocytes (Timmers *et al.*, 1996).

1.7.2 SM (Sec1/Munc like-) proteins

Although SM proteins are thought to be of great importance in the control of membrane fusion there is apparently conflicting data regarding the molecular basis of their function.

1.7.2.1 Original identification

The first SM protein, called Unc-18, to be discovered was found by screening *C. elegans* for mutations that caused an uncoordinated phenotype (Brenner, 1974). A subsequent screen in yeast for genes involved in the secretory pathway found a homologue to Unc-18 which was called Sec1(Novick *et al.*, 1980). A homologue of Sec1 has been found in *Drosophila,* this is called ROP (Harrison *et al.*, 1994). A neuronal homologue to Unc-18 was also discovered which encoded a protein of 67 kDa, which was found to bind stably to syntaxin 1 (Hata *et al.*, 1993;Garcia *et al.*, 1994). This homologue has subsequently been called Munc 18a.

SM proteins comprise a family of hydrophilic proteins, which span 60 to 70 kDa in size. Members of this protein family are found either within the cytosol of cells or attached to membranes through a high affinity interaction with their cognate syntaxin. In mammalian cells seven members of the SM family have been identified Munc 18a, -b, -c, Sly1p, mVps45, mVps33a and mVps33b (Dulubova *et al.*, 2003). Sequence homology is observed throughout the coding sequence suggesting that no one domain of the SM proteins is critical for function, compared with SNAREs where homology is only strongly observed within the SNARE domain (Halachmi and Lev, 1996).

1.7.2.2 Role of SM proteins in fusion

Although it is thought that all intracellular trafficking events require the participation of a member of the SM family, their exact role in membrane fusion has remained a contentious issue due to apparently conflicting data. While some studies suggest a positive role in membrane fusion others suggest that SM proteins play a negative role.

Null mutations of SM proteins in mice (Munc 18a), *C. elegans* (UNC-18), *S. cerevisiae* (Sec1p, Vps33p, Vps45p, Sly1p) and *Drosophila* (Rop) all led to a blockage of membrane fusion which suggests that SM proteins are essential for membrane fusion and play a positive role in fusion (Novick *et al.*, 1980, Banta *et al.*, 1990, Ossig *et al.*, 1991;Harrison *et al.*, 1994;Cowles *et al.*, 1994;Verhage *et al.*, 2000;Weimer *et al.*, 2003). This positive role has been supported by a recent *in vitro* fusion experiment which showed that fusion was stimulated on addition of the cognate SM protein (Scott *et al.*, 2004).

However, there is also data which points towards a negative role for SM proteins in exocytosis. Mutations in *Drosophila* syntaxin 1a which disrupt the interaction with the SM protein ROP increased the exocytosis of neurotransmitters supporting an inhibitory role for SM proteins in membrane fusion (Wu *et al.*, 1999). Munc 18a has also been shown to bind to a closed form of syntaxin 1a (Misura *et al.*, 2000b), which prevents the formation of SNARE complexes *in vitro* (Yang *et al.*, 2000) which further supports a negative role of SM proteins in SNARE complex formation and fusion.

It therefore remains to be established whether SM proteins play a positive or negative role in membrane fusion.

1.7.2.3 Munc 18 isoforms

The SM protein Munc 18a was found to be exclusively expressed in neuronal tissue (Hata *et al.*, 1993;Pevsner *et al.*, 1994b). Following the identification of Munc 18a two homologues were discovered. Munc 18b (or Munc 18-2) was identified by homology to Munc 18a (Hata and Sudhof, 1995). Unlike Munc 18a it was expressed in non-neuronal tissues (Hata and Sudhof, 1995). Using GST pull downs and a yeast two hybrid approach it was shown that both Munc 18a and Munc 18b could bind syntaxin 1, 2 and 3 but neither had an affinity for syntaxin 4 (Hata and Sudhof, 1995;Tellam *et al.*, 1997). Munc 18c was identified from a 3T3-L1 adipocyte cDNA library by homology to Munc 18a (Tellam *et al.*, 1995). This isoform of Munc 18 has been shown to bind to both syntaxin 2 and syntaxin 4 through GST pull downs (Tellam *et al.*, 1997). Both syntaxin 4 and Munc 18c are ubiquitously expressed and have both been implicated in tissues which regulate whole body glucose homeostasis, namely adipocytes, muscle and pancreatic β-cells.

1.7.2.4 Munc 18c

Like other studies on SM proteins, the role of Munc 18c in insulin-stimulated Glut4 translocation is highly contentious. Some data points towards a positive role for the SM protein in the fusion of Glut4 storage vesicles to the plasma membrane while other data supports a negative role.

While overexpression of Munc 18c in 3T3-L1 adipocytes inhibited insulin-stimulated Glut4 translocation and insulin-stimulated glucose transport, the overexpression of Munc

18b had no effect (Thurmond *et al.*, 1998;Tamori *et al.*, 1998). The overexpression of Munc 18c in transgenic mice also led to impaired glucose tolerance and glucose uptake into adipocytes (Spurlin *et al.*, 2003). The downregulation of Munc 18c by tetracycline repression of the transgene in these mice, or coexpression of syntaxin 4 normalised the response of these animals to glucose (Spurlin *et al.*, 2003). Together these data suggest that this SM protein has a negative regulatory role in Glut4 translocation to the cell surface.

Other studies however, have supported a positive role for this SM protein in the translocation of Glut4. The microinjection of a peptide from the sequence of Munc 18c, which was shown to compete for binding of Munc 18c to syntaxin 4, led to an inhibition of GSV fusion to the plasma membrane and an accumulation of these vesicles under the cell surface suggesting that this interaction is need for fusion of GSV to the cell surface (Thurmond *et al.*, 2000). This study has been supported by data from knock out mice. Homozygous knock-outs of Munc 18c are embryonically lethal, while heterozygote mice are viable (Oh *et al.*, 2005;Kanda *et al.*, 2005), supporting an essential role for this SM protein. In addition, heterozygous mice show an impaired ability to translocate Glut4 in response to insulin along with a reduced ability to secrete insulin from islet cells in response to insulin suggesting that this protein is a positive regulator of both of these fusion events (Oh *et al.*, 2005).

In contrast to the above, another study using adipocytes from mesenchymal embryonic fibroblasts from homozygote mice embryos showed that in these cells Glut4 translocation, measured by surface exposed Glut4 and 2-deoxyglucose uptake, is enhanced at suboptimal insulin doses in the absence of Munc 18c suggesting that Munc 18c may play a negative regulatory role in Glut4 translocation (Kanda *et al.*, 2005). However, at higher optimal insulin doses the absence of Munc 18c appeared to have no effect on Glut4 translocation (Kanda *et al.*, 2005).

The role of Munc 18c in Glut4 translocation is therefore still a contentious issue.

1.7.3 Rab protein family

The Rab family is the largest family in the Ras superfamily of small GTPases (20 to 30 kDa), being comprised of 60 family members in humans (Bock *et al.*, 2001). Rabs are known to be key regulators of vesicular traffic, and are located at distinct locations along

the exocytic and endocytic pathways of the cell, making them ideal candidates to regulate the specificity of fusion.

Rab proteins exist in two forms, one GTP bound which is considered to be active and interacts with downstream effectors, and one GDP bound which is considered to be inactive. A class of proteins known as guanine nucleotide exchange factors (GEFs) catalyse nucleotide exchange from the GDP to the GTP bound state, while another family of proteins the GTPase activating proteins (GAPs) catalyses GTP hydrolysis from the GTP to the GDP bound state. While GTP-bound Rabs are membrane bound, upon GTP hydrolysis Rabs are released from the membrane and associate with Rab GDP-dissociation inhibitor (GDI) in the cytosol (Novick and Zerial, 1997).

Several studies have demonstrated interactions between Rab proteins and members of the SNARE machinery or the SM family. In yeast, the delivery of Golgi-derived secretory vesicles to the plasma membrane depends on the Rab Sec4p that is known to interact with the large multi-protein tethering complex, the Exocyst (Guo *et al.*, 1999b). An interaction between the yeast t-SNARE Sed5p and the Rab-like GTPase Ypt1p has been shown (Lupashin and Waters, 1997). The data on this interaction suggested that this Rab reduced the amount of Sly1p, a member of the SM protein family, binding to Sed5p and facilitated the formation of v-/t-SNARE complexes (Lupashin and Waters, 1997) suggesting a key role for Rab proteins in the regulation of this fusion event. Recently it has been suggested that Rab proteins may displace SM proteins from syntaxin (Misura *et al.*, 2000a), whether Rab 4 displaces Munc 18c from syntaxin 4 is yet to be assessed.

Rab 4 has been suggested to play a role in Glut4 vesicular trafficking in adipocytes (Cormont *et al.*, 1996;Vollenweider *et al.*, 1997). Rab 4 has recently been shown to directly interact with syntaxin 4 which was modulated by the nucleotide bound state of Rab 4 (Li *et al.*, 2001). Whether Rab 4, or any other Rab family member, plays a role in regulating the fusion facilitated by syntaxin 4, SNAP23 and VAMP2 remains to be established.

1.7.4 Tomosyn

Tomosyn was first identified as a SNARE binding protein through its interaction with syntaxin 1A in neuronal tissues (Fujita *et al.*, 1998). The N-terminal of tomosyn is made up of WD-40 repeats the function of which are unknown (Hatsuzawa *et al.*, 2003). The C-

terminal of tomosyn contains a VAMP-like coiled coil domain through which it binds syntaxin (Fujita *et al.*, 1998).

Using a cell free system to study fusion, consisting of fusion competent secretory granules attached to isolated plasma membrane from PC12 cells, it was demonstrated that Tomosyn is inhibitory to exocytosis (Hatsuzawa *et al.*, 2003). The crystal structure of the SNARE motifs of syntaxin 1A, N/C-terminal portions of SNAP25 and the VAMP-like motif of tomosyn to 2.0-Å resolution revealed an overall structure extremely similar to the structure of the ternary complex of SNARE domains from the synaptic SNAREs (Pobbati *et al.*, 2004). However, this complex is less stable as it is not resistant to SDS (Pobbati *et al.*, 2004). Using the differential SDS resistance of the two complexes and fluorescently labelled proteins the authors demonstrated that prebinding of tomosyn's R-SNARE motif to the binary complex of syntaxin 1A/SNAP25 prevents the binding of VAMP2 while the prebinding of VAMP2 prevents the binding of Tomosyn (Pobbati *et al.*, 2004). This data suggests that Tomosyn plays a key role in regulating the formation of the ternary complex and thus membrane fusion.

Tomosyn was pulled out of a yeast two-hybrid screen using SNAP23 as bait (Masuda *et al.*, 1998). Tomosyn has 3 splice variants, s-, m- and b- (Yokoyama *et al.*, 1999). Full-length b-tomosyn has been shown to bind to syntaxin 4, more weakly to SNAP23 and with much higher affinity to the binary complex of syntaxin 4 and SNAP23 (Widberg *et al.*, 2003). This binding is facilitated through the same VAMP-like domain that facilitates binding to syntaxin 1A (Widberg *et al.*, 2003). The overexpression of b-tomosyn in 3T3-L1s led to a 40-50 % reduction in the ability of insulin to stimulate the translocation GFP-Glut4 to the cell surface as judged by cell surface staining and subcellular fractionation (Widberg *et al.*, 2003). The distribution of Tomosyn in the cell was unaffected by insulin stimulation, and although it is predominantly found in the cytosol of 3T3-L1s, it is also found in the LDM fraction and at the plasma membrane where it could interact with the fusion machinery responsible for exocytosis (Widberg *et al.*, 2003). This data suggests that Tomosyn has a negative effect on the fusion of Glut4 storage vesicles to the plasma membrane. Tomosyn was previously shown to compete with Munc 18a for binding to syntaxin 1A (Fujita *et al.*, 1998), however it appears that in the case of syntaxin 4, Munc 18c and tomosyn are able to bind simultaneously (Widberg *et al.*, 2003).

1.7.5 Synip

Synip, short for syntaxin 4 interacting protein, was isolated in a yeast two-hybrid screen of a 3T3-L1 adipocyte cDNA library using the cytoplasmic portion of syntaxin 4 as bait and was shown to be expressed in all insulin-responsive tissues (Min *et al.*, 1999). Synip binds specifically to the syntaxin isoform syntaxin 4 (Min *et al.*, 1999). The binding of synip to syntaxin 4 does not effect the binding of SNAP23 but does compete for binding of the t-SNARE complex to VAMP2 to form the ternary complex (Min *et al.*, 1999). Insulin stimulation was shown to decrease the binding of full-length synip to syntaxin 4 (Min *et al.*, 1999). The overexpression of full-length synip had no effect on insulin stimulated glucose uptake or Glut4 translocation to the plasma membrane (Min *et al.*, 1999). Synip is however, considered to be a negative regulator of Glut4 translocation in adipocytes since overexpression of the carboxy-terminal domain that does not dissociate from syntaxin 4 upon insulin stimulation inhibited Glut4 translocation and glucose uptake (Min *et al.*, 1999).

The insulin stimulated dissociation of synip from syntaxin 4 has subsequently been shown to be sensitive to wortmannin treatment, while expression of a dominant negative CAP was without effect, suggesting that the classical insulin-signalling pathway is responsible for the dissociation observed upon insulin stimulation (Yamada *et al.*, 2005). *In vitro* synip is phosphorylated on ser 99 by PKBβ and this phosphorylation is thought to control dissociation from syntaxin 4 (Yamada *et al.*, 2005). While it has been suggested from data in cells expressing a mutated synip, with this serine replaced with a phenylalanine, that this phosphorylation played a positive role in insulin stimulated glucose uptake (Yamada *et al.*, 2005) a subsequent study which mutated this serine to a alanine found no effect (Sano *et al.*, 2005) suggesting this result may be an artefact.

1.7.6 Protein Kinases

A plausible way that insulin signalling could influence fusion is through the activation of protein kinases that may phosphorylate the fusion machinery. A number of kinases have been shown to phosphorylate the SNAREs involved in Glut4 exocytosis as outlined in Table 1.1.

Protein	Kinase	Effect of phosphorylation	Reference
Syntaxin 4	PKC	Reduced binding to SNAP23	Chung *et al.*, 2000
	PKA	Reduced binding to SNAP23	Foster *et al.*, 1998
	casein kinase II	Not determined	Foster *et al.*, 1998
SNAP23	PKC	Reduced binding to syntaxin 4	Polgar *et al.*, 2003
	SNAK	Not determined	Cabaniols *et al.*, 1999
VAMP2	PKCζ	Not determined	Braiman *et al.*, 2001
Munc 18c	PKC	Reduced binding to syntaxin 4	Schraw *et al.*, 2003

Table 1.1: Protein kinases that phosphorylate the SNAREs involved in Glut4 exocytosis (Adapted from Weinberger and Gerst, 2004))

PKC is an important downstream effector involved in the classical insulin-signalling pathway. In insulin responsive tissues the activation of PI3K by the classical insulin signalling pathway leads to the activation of PKCζ which has been shown to be important in the subsequent transport of the Glut4 isoform (Standaert *et al.*, 1997). In myotubes the overexpression of PKCζ, through the use of an adenovirus, increased basal glucose transport and translocation of Glut4 to the plasma membrane, while the expression of a

dominant negative mutant had no effect on basal glucose transport but prevented insulin stimulated glucose transport and Glut4 translocation supporting the key role of this enzyme in insulin stimulated glucose transport (Braiman *et al.*, 2001). Upon insulin stimulation PKCζ was shown to associate with the Glut4 containing compartment (Braiman *et al.*, 2001). Insulin stimulation of myotubes was shown, through probing immunoprecipitations of VAMP2 with a anti-phosphoserine antibody, to lead to phosphorylation of serine residues within VAMP2 (Braiman *et al.*, 2001). The overexpression of PKCζ led to a more rapid phosphorylation of VAMP2 while the overexpression of a dominant negative version of PKCζ prevented any insulin stimulated serine phosphorylation of this SNARE (Braiman *et al.*, 2001). PKCζ was also shown to directly phosphorylate VAMP2 using an *in vitro* kinase assay (Braiman *et al.*, 2001). The consequences of this phosphorylation await further investigation.

Another important downstream effector of PI3K is PKB. Three isoforms of PKB have been identified, called PKBα, PKBβ and PKBγ. PKB was substantially recruited to the Glut4 containing compartment following insulin stimulation of 3T3-L1 adipocytes (Calera *et al.*, 1998). Several studies have shown that activation of PKB isoforms is involved in Glut4 translocation. The microinjection of 3T3-L1 adipocytes with a PKB substrate peptide or antibody specific for PKBα and PKBβ led to an inhibition of insulin-stimulated Glut4 translocation measured as cell surface Glut4 (Hill *et al.*, 1999). However, this study did not elucidate exactly which isoform is involved in Glut4 translocation. Using siRNA to reduce the expression level of PKB isoforms it was shown that knock down of PKBα only gave a small inhibition while, knock down of PKBβ by 70 % inhibited of Glut4 translocation by 50 % (Jiang *et al.*, 2003). Isoform specific knock down of PKB was also carried out by another group and like the previous study showed that while PKBα knock down did slightly inhibit both insulin stimulated 2-deoxyglucose uptake and Glut4 translocation in 3T3-L1 adipocytes the effect was much greater on knock down of PKBβ (Katome *et al.*, 2003). This study also showed that the knock down of PKBγ had no effect on either insulin-stimulated glucose uptake or Glut4 translocation (Katome *et al.*, 2003). Genetic ablation of PKBβ in mice has been shown to lead to insulin resistance (Cho *et al.*, 2001a) while mice deficient in PKBα demonstrated no defect in clearance of blood glucose during a glucose tolerance test (Cho *et al.*, 2001b). These results were supported by a study using adipocytes differentiated from mouse embryos with homozygous deletion of PKBα and PKBβ (Bae *et al.*, 2003). The main PKB isoform involved in regulating insulin stimulated Glut4 translocation is therefore thought to be PKBβ.

PKBβ has been shown to be capable of phosphorylating proteins on immunoabsorbed Glut4-containing vesicles form rat adipocytes but whether its substrates include VAMP2 is yet to be confirmed (Kupriyanova and Kandror, 1999). Recently an assay has been developed to study the fusion of Glut4 storage vesicles to the plasma membrane *in vitro* (Koumanov *et al.*, 2005). This assay allows the study of the fusion of these two membranes in isolation from effects on other Glut4 trafficking events within the cell. The assay involves isolated plasma membrane reconstituted into liposomes, isolated GSVs and fusion is monitored by a modified glucose substrate. Using this assay it was shown that fusion between the reconstituted plasma membrane and the isolated GSVs was dependent on PKBβ (Koumanov *et al.*, 2005). Fusion was greatly increased when plasma membrane from insulin treated cells was used as opposed to basal plasma membrane suggesting that the target of the insulin signalling pathway is a component of the plasma membrane in adipocytes and demonstrating this fusion event is indeed regulated by insulin signalling (Koumanov *et al.*, 2005).

In addition to Synip, as mentioned above, two other substrates of PKB have been identified which have been implicated in insulin-regulated Glut4 trafficking. One of these substrates AS160, was initially isolated from pull down experiments in 3T3-L1 adipocytes using a phospho-PKB substrate antibody raised against the PKB consesus phosphorylation site (Kane *et al.*, 2002). In addition to a GAP domain, AS160 has 6 PKB phosphorylation consensus sites (Sano *et al.*, 2003). Five of these consensus sites have been shown to be phosphorylated in response to insulin, namely Ser318, Ser570, Ser588, Thr642 and Thr571 (Sano *et al.*, 2003). The phosphorylation of four of these sites has been implicated in insulin-regulated Glut4 trafficking, since overexpression of a mutant of AS160 with these sites mutated to alanines led to an inhibition of Glut4 translocation in 3T3-L1 adipocytes (Sano *et al.*, 2003). RNAi of AS160 in 3T3-L1s has provided further support for its role in insulin-stimulated Glut4 translocation, since reduced cellular levels of AS160 led to an increase in Glut4 translocation to the plasma membrane (Larance *et al.*, 2005). This study also found that upon insulin-stimulation AS160 dissociates from Glut4 containing vesicles (Larance *et al.*, 2005). Although AS160 has been shown to have GAP activity against a subset of Rab proteins, none of these Rabs have so far been shown to participate in insulin-stimulated Glut4 translocation (Miinea *et al.*, 2005). The precise molecular role for AS160 in insulin-stimulated Glut4 translocation is therefore yet to be established.

The second potential PKB substrate to be identitified, PIKfyve (Phosphoinositide kinase for five position containing a Fyve finger), was characterised using a proteomic approach with a antibody raised against the PKB consensus phosphorylation site (Berwick *et al.*, 2004). PIKfyve is phosphorylated in respose to insulin, on Ser318 (Berwick *et al.*, 2004).. A kinase dead version of PIKfyve inhibited the translocation of Glut4 in 3T3-L1 adipcytes (Ikonomov *et al.*, 2002). In addition, the overexpression of a mutant of PIKfyve, with Ser318 mutated to an alainine, was shown to increase Glut4 translocation in 3T3-L1 adipcytes (Berwick *et al.*, 2004). These data support a role for this PKB substrate in insulin-stimulated Glut4 translocation.

1.8 Membrane domains and Insulin signalling

It is now widely believed that instead of a sea of lipids the plasma membrane is organised into a number of different discrete domains, and that these domains may play an important role in a number of cellular processes. Many proteins are thought to be confined to regions or domains within the plasma membrane that have a different lipid composition to the surrounding membrane.

The mammalian plasma membrane is made up of three main classes of lipids; glycerophospholipids, sphingolipids and cholesterol. The plasma membrane has been proposed to exist of domains enriched in cholesterol and saturated sphingolipids (sphingomylein and glycosphingolipids), termed lipid rafts, surrounded by a sea of membrane mainly made up of unsaturated glycerophospolipids (Simons and Ikonen, 1997). These domains have also been proposed to concentrate some proteins while excluding others in effect acting as signalling platforms (Simons and Ikonen, 1997). Cholesterol is known to interact more favourably with sphingolipids than glycerophospholids resulting in very tight packing between the molecules which gives the "liquid ordered state" in comparison to the bulk plasma membrane which exists in a "liquid disordered state" as shown in Figure 1.6.

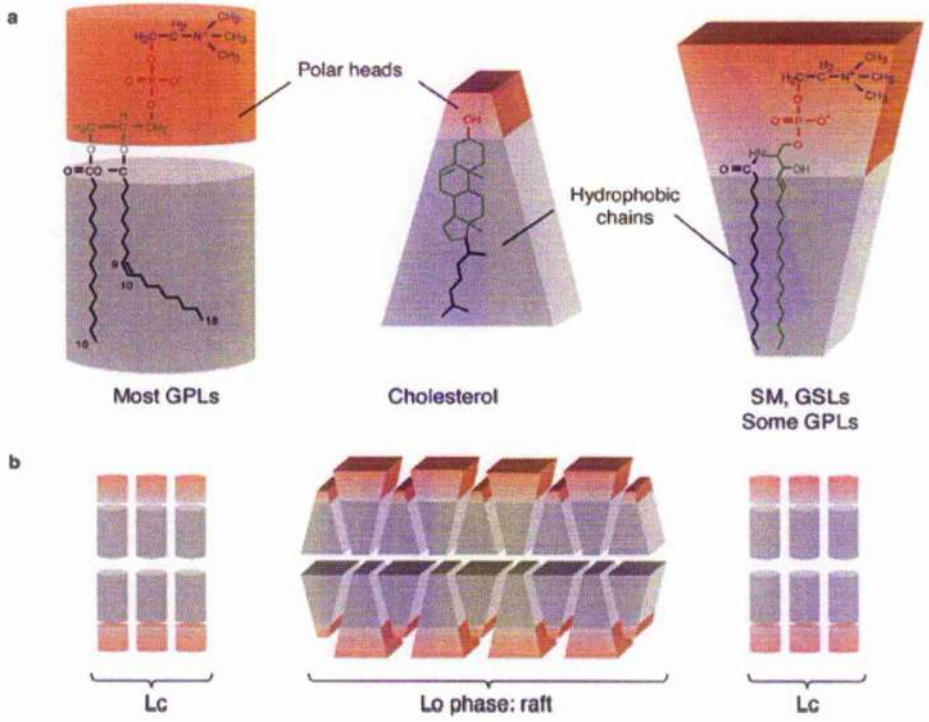


Figure 1.6: Proposed organisation of lipid raft domain

The different geometries of membrane lipids are thought to lead to the formation of domains within the bilayer known as lipid rafts. (A) Most glycerophospholipids (GPLs), which make up the bulk membrane, are cylindrical in shape. Cholesterol, Glycosphingolipids (GSLs) and sphingomyelin (SM) have a cone like shape. (B) Lipid raft domains are enriched in GSLs and cholesterol. The bulky head groups of the sphingolipids leave space to accommodate the tight packing of cholesterol which intercalates between the acyl chains. This tight packing gives rise to a "liquid ordered state" or Lo state in comparison to the bulk plasma membrane which exists in a "liquid-disordered state" or Lc state. (Taken from Fantini *et al.*, 2002)

1.8.1 Caveolae

Using electron microscopy Palade and Yamada independently identified morpholigiaclly distinct regions of the plasma membrane in two different cell types; endothelial cells and gall bladder (Palade, 1953;Yamada, 1955). These domains appeared as flask-like invaginations of the plasma membrane, and were termed caveolae by Yamada who thought they looked like small caves (Yamada, 1955). Caveolae are estimated to have a diameter of ~50 to 100 nm and have been found in abundance in many tissues. While individual caveolae are observed at the surface of the cell a number of caveolae can come together to form grape like structures.

Following the identification of caveolae a marker protein caveolin-1 was discovered (Rothberg *et al.*, 1992). Cells from Caveolin-1 knock out mouse lack the flask-like morphology of caveolae suggesting that this protein forms the architecture of these domains (Drab *et al.*, 2001). Caveolin has been shown to bind directly to cholesterol (Murata *et al.*, 1995), and the caveolin isoforms have been shown to homo- and hetero-oligomerise: these interactions are thought to be of fundamental importance to the morphology of caveolae (Sargiacomo *et al.*, 1995;Scherer *et al.*, 1997). Two other caveolin isoforms, expressed from separate genes, have since been identified, caveolin-2 and caveolin-3. All of the caveolin isoforms are integral membrane proteins with a mass of 21 to 25 kDa. While caveolin 1 and 2 are coexpressed in nearly all cell types (Scherer *et al.*, 1997), the expression of caveolin 3 is specific to muscle cells (Song *et al.*, 1996).

Research has demonstrated that many signalling proteins are present in caveolae and caveolae have subsequently been proposed to represent signalling platforms within the cell. These signalling proteins include GPCRs, heterotrimeric G proteins and nitric oxide synthase. Endothelial nitric oxide synthase (eNOS) has been shown to bind to caveolin-1 and this binding keeps eNOS in an inactive form, with the activation of eNOS being initiated by its dissociation from caveolin-1 (reviewed in Goligorsky *et al.*, 2002). In addition to eNOS a number of other proteins have been shown to bind directly to caveolin, including the EGF Receptor (Couet *et al.*, 1997b). The insulin receptor contains a binding motif, similar to that contained in the EGF receptor, for caveolin-1 suggesting it may be recruited to these domains (Couet *et al.*, 1997a). Although the insulin receptor has been immunolocalised to caveolae (Gustavsson *et al.*, 1999), a subsequent study failed to find the receptor within these domains (Souto *et al.*, 2003). While caveolin 1 deficient mice

display insulin resistance in adipose tissue (Cohen *et al.*, 2003), caveolin 3 deficient mice display insulin resistance in muscle (Oshikawa *et al.*, 2004) suggesting that the protein caveolin has an important role in insulin signalling.

In addition to their signalling role caveolae are thought to facilitate the transport of proteins into the cell. Caveolae are now known to pinch off into the cell. This is though to be facilitated by the large GTPase dynamin (Henley *et al.*, 1998;Oh *et al.*, 1998) and a direct interaction between dynamin 2 and caveolin has been observed (Yao *et al.*, 2005).

1.8.2 Lipid rafts

Due to their distinguishable morphology the existence of caveolae at the cell surface is widely accepted. The existence of membrane domains enriched in cholesterol and sphingolipids with no morphologically defined features by electron microscopy is more controversial (Munro, 2003). The existence of lipid raft domains was originally suggested following their isolation as detergent insoluble domains form cells lacking caveolae (Gorodinsky and Harris, 1995).

Lipid rafts are commonly biochemically isolated on the basis of their insolubility in ice-cold non-ionic detergent such as Triton X100, which is thought to be caused by the tight packing of their lipid constituents, and light buoyant density on sucrose gradient centrifugation (Chamberlain, 2004). Caveolae, discussed above, which are also purified using this technique due to their high cholesterol and sphingolipid content are considered to be a subset of the lipid raft domain.

Many proteins have been found to be associated with raft domains in mammalian cells. The addition of saturated acyl chains, such as myristate or palmitate, is thought to target proteins to lipid raft fractions as these are particularly well suited to the packing between lipids in these domains (Wang and Silvius, 2000). The recruitment of transmembrane proteins to rafts is yet to be characterised but it has been shown that the thickness of a liquid ordered domain is greater than that of the crystalline state suggesting the length of the transmembrane domain may play a role in directing proteins to lipid rafts (Gandhavadi *et al.*, 2002).

A recent proteomic analysis of detergent resistant domains suggests that signalling proteins may be enriched ten fold in raft domains compared to the rest of the plasma membrane

(Foster *et al.*, 2003). The recruitment of proteins involved in signalling cascades to lipid raft domains is thought to facilitate local and efficient interaction, which has led to lipid rafts being proposed as signalling platforms within the cell.

1.8.3 Insulin signalling and lipid raft domains

The spatial organisation of signalling molecules into areas of close proximity has been proposed to increase the specificity, efficiency and level of regulation of signalling cascades. It has therefore been postulated that as such areas of organisation lipid rafts may act as signalling platforms within the plasma membrane. Components of the insulin-signalling pathway have been localised to lipid raft domains and caveolae within adipocytes.

Using immunogold microscopy and subcellular fractionation it has been shown that a fraction of the insulin receptor itself colocalises with caveolae in adipocytes (Gustavsson *et al.*, 1999), although a subsequent study has questioned this data (Souto *et al.*, 2003). The insulin receptor has been shown to, most probably indirectly, phosphorylate the main marker protein of caveolae, caveolin (Mastick *et al.*, 1995;Kimura *et al.*, 2002) supporting the possibility of localisation within DRMs. The PI3K-independent insulin signalling pathway has also been extensively localised to lipid raft domains (reviewed in Saltiel and Pessin, 2003).

Recently it was observed that individual caveolae present on the plasma membrane of differentiated adipocytes are often arranged into ordered ring-like structures, and that these structures are associated with F-actin (Kanzaki and Pessin, 2002). TC10, a member of the PI3K-independent insulin signalling cascade, has been localised to these ring-like organisations (Watson *et al.*, 2001;Kanzaki and Pessin, 2002). This is of interest as Glut4 translocation has been shown to require actin remodelling (Kanzaki *et al.*, 2001), and TC10 is a member of the Rho family of GTPases which are involved in actin remodelling. These novel macrodomains are only found in differentiated adipocytes (Parton *et al.*, 2002). In response to insulin stimulation, Glut4 appears to be recruited to these cave like structures (Parton *et al.*, 2002). Although these macrodomains contain caveolin they also contain non-raft markers and clathrin coated pits (Parton *et al.*, 2002). Glut4 has previously been shown to translocate to caveolae upon insulin stimulation of adipocytes (Ros-Baro *et al.*,

2001;Karlsson *et al.*, 2002), but the relationship of caveolae to caves in this study is not clear.

1.8.4 Raft association of SNAREs involved in Glut4 translocation

SNARE proteins were first localised to detergent insoluble lipid raft domains in polarised Madin Darby Canine Kidney cells. In this cell type the t-SNARE syntaxin and the v-SNARE TI-VAMP were both shown to be present in the lipid raft fraction following Triton extraction and sucrose gradient centrifugation (Lafont *et al.*, 1999). The raft targeting of these proteins was suggested to facilitate the targeting of these SNAREs to the apical membrane of the cell (Lafont *et al.*, 1999). The association of SNAREs with lipid raft domains has subsequently been demonstrated in a number of other cell types, including endothelial cells (Schnitzer *et al.*, 1995) and rat brain synaptosomes (Gil *et al.*, 2005).

The components of the neuronal exocytic t-SNARE complex syntaxin 1A/SNAP25 and the v-SNARE VAMP2 have also been shown to partition into detergent insoluble domains in PC12 cells, a rat neuronal cell line (Chamberlain *et al.*, 2001). These findings led to an investigation into whether the SNAREs involved in Glut4 translocation to the plasma membrane are also associated with detergent insoluble domains. Using 3T3-L1 adipocytes it was demonstrated that both the t-SNAREs syntaxin 4 and SNAP23 and the v-SNARE VAMP2 did partition into such domains (Chamberlain and Gould, 2002). A substantial proportion of both of these t- SNAREs, roughly 70 % of SNAP23 and 35 % of syntaxin 4, were associated with detergent insoluble domains isolated from the plasma membrane of this cell type (Chamberlain and Gould, 2002). The distribution of the t-SNAREs within the plasma membrane seemed to be unaffected by insulin stimulation and depletion of cholesterol, by treatment with methyl-β-cyclodextrin, led to dispersion of these t-SNAREs through the membrane supporting the evidence they were associated to lipid raft domains (Chamberlain and Gould, 2002). Immuno-fluorescence showed that SNAP23 did not co-localise with caveolin-1, the caveolae marker protein in this cell type, suggesting that these domains are distinct from caveolae.

Proteins known to be regulators of SNARE function, including Munc18, Munc 13, αSNAP and NSF, were not found to be present in the detergent insoluble domains isolated from PC12 cells (Chamberlain *et al.*, 2001). Whether the exclusion of these proteins from lipid

raft domains plays a role in regulating the fusion SNAREs facilitate remains to be established.

1.9 Aims of this study

The basis of this study was to investigate the fusion facilitated by the exocytic SNAREs involved in Glut4 translocation, syntaxin 4, SNAP23 and VAMP2.

In Chapter 3 as a first step towards studying the fusion facilitated by these SNAREs an *in vitro* fusion assay was established using these SNAREs expressed in and purified from *E. coli* as recombinant proteins.

The exocytic SNAREs involved in Glut4 translocation have been localised to lipid raft domains in the insulin-responsive cell 3T3-L1 adipocytes. In Chapter 4 in order to investigate whether the lipids known to be enriched in such domains, sphingomyelin and cholesterol , influence the fusion facilitated by these SNAREs these lipids were introduced into the *in vitro* fusion assay established in Chapter 3. SNAP23 is known to be associated to the membrane through the addition of palmitate groups to a central cysteine rich domain. In order to purify palmitoylated SNAP23 for future experiments a recombinant baculovirus expressing the t-SNARE complex of syntaxin 4 and SNAP23 was also constructed in Chapter 4.

In the final results chapter, Chapter 5, the conformation of syntaxin 4 and its interaction with the SM protein Munc 18c was investigated. Like SNARE proteins SM proteins are known to be involved in every intracellular fusion event and the conformation adopted by syntaxins is thought to influence their mode of interaction with these regulatory proteins. In neuronal exocytosis syntaxin 1a is thought to be held in a closed conformation by its cognate SM protein Munc 18a preventing the formation of SNARE complexes. In chapter 5, we set out to investigate whether the interaction between syntaxin 4 and its cognate SM protein Munc 18c takes place through a similar mode.

Chapter 2

Materials and Methods

Materials

All materials used in this study were of high quality and were obtained from the following suppliers:

2.1 Materials

2.1.1 General reagents

Agar Scientific

Quantifoils

Amersham Biosciences UK limited, Little Chalfont, Buckinghamshire, UK

ECL western blotting detection agents

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

Glutathione Sepharose 4B

Anachem Ltd., Luton, Bedfordshire, UK

30 % acrylamide/bisacrylamide

Avanti polar lipids, Alabaster, USA

1,2-dioleoyl phosphatidylserine (DOPS)

Cholesterol

1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)

N-(Lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE)

(N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE)

Sphingomyelin

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

Biobeads

Bradford protein assay reagent

Eco-Pac disposable chromatography columns

Low molecular weight protein markers

N,N,N`,N`-teramethylethylenediamine (TEMED)

Calbiochem, Beeston, Nottingham, UK

4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF)

Fisher Scientific Ltd, Loughborough, Leicestershire, UK

Ammonium persulphate

Calcium chloride

Diaminoethanethanetera-acetic acid, disodium salt (EDTA)

Glycerol

Glycine

N-2-hydroxyethylpiperizine-N`-2-ethanesulphonic acid (HEPES)

Hydrochloric acid

Isopropanol

Methanol

Potassium Chloride

Potassium dihydrogen orthophosphate

Sodium chloride

Sodium dihydrogen orthophosphate dihydrate

Sodium dodecyl sulphate

Invitrogen Ltd, Paisley, UK

Cellfectin Reagent

DH10 Bac™ *E. coli*

Graces insect cell medium

PCRII-TOPO™ TA cloning kit

pFastBac™ Dual

10000 U/ml penicillin, 10000 U/ml streptomycin

Sf9 cells SFM (serum free media) adapted

Sf900-II media

SOC Media

Kodak Ltd, Hemel Hempstead, Hertfordshire, UK

X-ray film

Melford Labs

Micro agar

Terrific broth

Tryptone

Yeast extract

Merck Bioscience, Beeston, Nottingham, UK

Rosetta™ (DE3) pLysS Singles™ Competent cells

MWG-Biotech, Germany

Oligonucleotide orders

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

Pre-stained broad range protein marker (6-175 Kda)

Premier Brands UK, Knighton, Adbaston, Staffordshire, UK

Marvel powdered milk

Priceton Separations, Adelphia, NJ

Chymotrypsin (Bovine) Sequencing Grade Modfied

Promega, Southampton, UK

All restriction enzymes

Deoxynucleotides (dNTPs)

Pfu polymerase

Taq polymerase

Qiagen, Crawley, West Sussex, UK

Nickel NTA-agarose (Ni-NTA agarose)

QIAfilter™ plasmid maxiprep kit

QIAprep™ spin miniprep kit

Qiagen gel purification kit

Roche, Bassel, Switzerland

Complete™ and complete™ EDTA-free Protease Inhibitor Tablets

Schleicher & Schell, Dassel, Germany

Nitrocellulose membrane (pore size: 45 µM)

Spectrum laboratories

Float-a-lyzer, 3 ml 10000 kDa MWCO

TAGN, Newcastle, UK

Oligonucleotide orders

Whatman International Ltd, Maidstone, UK

Whatman No. 2 filter paper

Whatman No. 3 filter paper

Whatman 3 mm filter paper

All other chemicals used were supplied from Sigma Chemical company limited, Poole, Dorset, UK unless otherwise stated.

2.1.2 Primary antibodies

Syntaxin 4 and SNAP23 primary antibodies were both purchased from synaptic systems and were raised in rabbits.

2.1.3 *Escherichia coli* (*E. coli*) strains

All bacterial strains used are modifications of *E coli.*

DH5α	F⁻ ϕ80d*lac*Z ΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_{k-}, m_{k+}) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1
TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcrBC*) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
BL21 DE3	F⁻ *ompT hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3)
Rosetta™ (DE3) pLysS Singles™	F⁻ *ompT hsd*S_B(r_B^- m_B^-) *gal dcm lacY1* (DE3) pLysSRARE (*argU, argW, ileX, glyT, leuW, proL*) (Cm^R)
DH10 Bac™	F⁻ *mcr*A (*mrr-hsd*RMS-*mcr*BC) ϕ80lacZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G /pMON14272 / pMON7124

2.1.4 General solutions

A200	25 mM HEPES pH 7.4, 200 mM KCl, 10 % (w/v) glycerol, 2 mM DTT pH 7.4

DNA loading buffer	40% (w/v) Ficoll, 0.25% bromophenol blue
HIS-purification buffer	100 mM HEPES, 200 mM KCL, 5 mM imidazole, 2 mM β-mercaptoethanol pH8.0
LB media	0.1 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract, 0.1 % (v/v) glycerol
GST-purification buffer	100 mM HEPES, 500 mM NaCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol pH 7.4
Phosphate buffered saline (PBS)	136 mM NaCl, 10 mM NaH_2PO_4, 2.5 mM KCL, 1.8 mM KH_2PO_4 pH 7.4
PBS-T	PBS, 0.02 % Tween-20
SDS-PAGE electrode buffer	25 mM Tris base, 190 mM glycine, 0.1 % (w/v) SDS
SDS-PAGE sample buffer	93 mM Tris. HCl pH 6.8, 20 mM dithiothreitol (added immediately before use), 10 % (w/v) glycerol, 2% (w/v) SDS, 0.002 % (w/v) bromophenol blue.
SOC media	2 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
TAE	40 mM Tris-acetate, 1 mM EDTA pH 7.8
Terrific broth	1.2 % (w/v) Tryptone, 2.4 % (w/v) Yeast extract, 0.4 % (v/v) glycerol, 2.3 % (w/v) KH_2PO_4, 12.5 % (w/v) K_2HPO_4
Transfer buffer	25 mM Tris base, 192 mM glycine, 20 % (v/v) methanol

2.2 General laboratory procedures

2.2.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on the basis of size using SDS-PAGE. SDS-PAGE was carried out using Bio-Rad mini-PROTEAN III apparatus. The percentage of acrylamide in each gel ranged from 8 to 15% depending on the molecular weight of the protein of interest. This was overlaid with 100 % isopropanol, leaving enough room for the stacking gel.

Protein samples were solubilised in SDS-PAGE sample buffer and incubated in a heat block at 95 °C for 5 minutes. Gels were run in SDS-PAGE electrode buffer, at a constant voltage of 80 volts through the stacking gel, which was increased to 140 volts through the resolving gel. Gels were electrophoresised until the dye front had reached the desired position or the pre-stained broad range markers had adequately separated.

2.2.2 Western Blotting

Following SDS-PAGE proteins were transferred onto nitrocellulose membrane for immunodetection analysis. Gels were carefully removed from the plates. A sandwiched arrangements of components, individually soaked in transfer buffer were made as follows from bottom to top: a sponge pad, a layer of whatman 3 mm filter paper, nitrocellulose paper (pore size 0.45 μm), polyacrylamide gel (electrophoresed to resolve proteins as outlined in section 2.2.1), a layer of Whatman 3 mm filter paper, a sponge pad. The sandwich was placed in a cassette and slotted, bottom nearest to cathode, into a Bio-Rad mini trans-blot tank filled with transfer buffer. Transfer of proteins was performed at room temperature for 3 hours at a constant current of 250 mA or overnight at 40 mA. Efficiency of transfer was determined by staining the nitrocellulose with Ponceau S solution, which was then removed by washing in PBS-T, prior to blocking.

2.2.3 Immunodetection of proteins

Following western blotting, as outlined in section 2.2.2, proteins were detected by Enhanced Chemical Luminescence. The nitrocellulose membrane was removed from the sandwich and washed with PBS-T. The membrane was incubated with 5% (w/v) dried non-fat milk made up in PBS-T for 1 hour to block non-specific binding sites. Primary

antibodies were diluted in 1 % (w/v) non-fat milk/PBS-T at the appropriate dilution. The membrane was incubated with the primary antibody for at least 1 hour at room temperature while shaking. Membrane was washed three times over 30 minutes with PBS-T and incubated with the appropriate secondary antibody (HRP-linked Ig) at a 1:1000 dilution for 1 hour at room temperature. The membrane was then washed three times over 30 minutes with PBS-T.

Immunolabelled proteins on western blots were visualised using HRP-conjugated secondary antibody and the ECL system (Amersham, UK). ECL reagents 1 and 2 were mixed in a 1:1 ratio and the membrane immersed in this mix for 1 minute. Excess ECL was removed and the membrane exposed to Konica X-ray film in a light proof cassette.

2.2.4 *Coomassie blue staining of SDS polyacrylamide gel*

A Coomassie blue stain solution was produced by mixing 10 mls of methanol:water at a ratio of 1:1 with 90 mls of distilled water and 0.25g of Brilliant Blue R. This was thoroughly stirred and then filtered through whatman No 2 filter paper. Gels were stained by soaking in the Coomassie blue stain solution at least 1 hour. Gels were then destained in the same solution as the Coomassie blue stain but without the addition of Brilliant Blue R, for 2 hours to overnight depending on the resolution required.

2.3 General Molecular Biology

2.3.1 *Amplification of DNA by Polymerase Chain Reaction (PCR)*

Appropriate forward and reversed primers containing the desired restriction sites were designed and ordered from TAGN (Newcastle, UK) or MWQ (Ebersberg, Germany). All PCRs were carried out in thin walled tubes in a thermocycler.

Plasmid DNA purified using a QIAprep™ spin miniprep kit, as outlined in section 2.3.10, was generally used as the template. Primer stocks were made up in nuclease free water at 100 pmol/µl.

Reactions were set up on ice typically as follows:

Nuclease-free water	37 µl
10 x DNA polymerase buffer (with $MgCl_2$)	5 µl
Template DNA (50-100 ng)	1 µl
Forward primer (4 pmol / µl)	2.5 µl
Reverse primer (4 pmol / µl)	2.5 µl
dNTP (10 mM each dCTP, dGTP, dATP, dTTP)	1 µl
Pfu Turbo DNA polymerase	1 µl
Total volume	50 µl

The general conditions used for PCR are outlined below:

95 °C	2 min	
94 °C	30 sec	25 cycles
55 °C	30 sec	
72 °C	2min/Kb	
72 °C	10 min	
4 °C	Hold	

The annealing temperature of 55 °C was adjusted depending on the melting temperatures of the primers involved. An aliquot of the PCR mix, typically 10 µl, was run on an agarose gel to check for the presence of a product of the correct size as outlined in section 2.3.3.

2.3.2 Site-directed mutagenesis

This was essentially carried out according to the QuikChange® method (Stratagene). The exchange of one base to another was performed by using synthetic oligonucleotides which were designed with the desired mutation in the middle of the primer. Plasmid DNA

purified using a QIAprep™ spin miniprep kit was generally used as the template. The reaction mixture used for site directed mutagenesis is as follows:

Nuclease-free water	37 µl
10 x DNA polymerase buffer (with $MgCl_2$)	5 µl
Template DNA (5 - 50 ng)	1 µl
Forward primer (4 pmol / µl)	2.5 µl
Reverse primer (4 pmol / µl)	2.5 µl
dNTP (10 mM each dCTP, dGTP, dATP, dTTP)	1 µl
Pfu Turbo DNA polymerase	1 µl
Total volume	50 µl

The tubes were flick mixed and briefly spun in a microfuge.

The thermo cycling conditions used for site directed mutagenesis were as follows:

95°C	1 min	
94°C	30 sec	16-18 cycles
55°C	30 sec	
68°C	1min/Kb	
68°C	10 min	
4°C	Hold	

After the reaction was complete the mixture was cooled on ice for 10 minutes prior to the addition of 1µl of *Dpn* I (10 units/µl). The reaction was then incubated for 1 hour at 37 °C in order to cut the parental DNA. A small aliquot of the mixture, 1-2 µl, was transformed into DH5α competent cells. The plasmids were isolated from single colonies as outlined in 2.3.10 and the mutations were confirmed by DNA sequencing as outlined in 2.3.12.

2.3.3 Agarose Gel electrophoresis

Agarose gels were made with 1 % agarose in TAE buffer. For a standard 1 % agarose gel 0.5g of agarose added to 50 ml of TAE buffer and this was boiled using a microwave. The agarose solution was then allowed to cool to roughly 50 ^{0}C and 1µl of ethidium bromide (10 mg/ml) was added to this. The agarose solution was then poured into a horizontal gel plate containing an appropriate comb and tape at each end. The agarose was allowed to fully set and then the tape and comb were removed and the gel plate transferred to a DNA gel tank for loading and running of DNA samples.

DNA samples were prepared by adding 6x DNA load dye and loaded into the appropriate lanes. Either a 1kb or a 100 bp DNA ladder was run alongside these samples depending on the expected size of product. The gel was run at 80 volts until the dye front was at a relevant point and then the gel was viewed on a transilluminator. A photographic record was taken of each DNA gel.

2.3.4 Purification of PCR product

Following electrophoresis as described in section 2.3.3 agarose gels were examined under UV light for the presence correctly sized product. Bands of the correct size were excised with a clean scalpel blade. PCR products were then purified using the Qiagen gel purification kit following the manufacturers instructions. Briefly 600 µl of solublisation Buffer QC was added to the gel slice, which was subsequently incubated for 10 minutes or until the agarose had completely dissolved at 50 ^{0}C. Following this incubation 200 µl of isopropanol was added and this mixture was transferred to a QIAspin column. This was spun at 14,000 xg for 1 minute. The column was then washed with 750 µl of Buffer PE. The column was then spun for 1 minute at 14,000 xg and any remaining traces of ethanol removed by a final spin at 14,000 xg for 2 minutes. The DNA was eluted in a final volume of 50 µl of nuclease-free water, which was added to the centre of the column and incubated for 1 minute prior to centrifugation for 1 minute at 14,0000 xg.

2.3.5 Restriction digestion

Plasmid or PCR product were cut with a pair of restriction enzymes either to identify DNA with the expected restriction pattern or to purify fragments prior to ligation. The buffer

used for restriction digest was selected on the basis that both enzymes retained 75 -100 % activity in this buffer. Restriction digests were set up as follows

DNA (0.5 - 1 μg)	5 μl
10 X Buffer	1μl
Restriction enzyme 1	1μl
Restriction enzyme 2	1μl
Sterile nuclease free water	2 μl

The tubes were flick mixed and briefly centrifuged prior to incubation at 37 °C for 2 hours. Following digestion products were analysed using agarose gel electrophoresis as outlined in section 2.3.3.

2.3.6 TA Cloning

In order to clone PCR products into pCRII-TOPO a 3` A overhang must be incorporated into the purified *Pfu* PCR product (the pCRII-TOPO vector contains a complimentary 5` T overhang). This was achieved by incubation with *Taq* polymerase. Incubations were set up as follows:

Gel purified DNA	20 μl
10 X Mg free Buffer	2 μl
$MgCl_2$	1.2 μl
Taq polymerase	0.4 μl
dNTPs	0.4 μl

The tubes were flick mixed and briefly centrifuged prior to incubation at 72 °C for 20 minutes.

TA cloning was performed using the pCRII-TOPO kit (Invitrogen), according to the manufacturers instructions. Briefly 2 μl of purified *Taq* treated PCR product was added to 0.5 μl of pCRII-TOPO vector in a sterile eppendorf tube and the overhangs allowed to

anneal at room temperature for 5 minutes. The reaction was terminated by the addition of 0.5 µl of salt solution. The whole reaction was then used to transform chemically competent TOP10 cells as outlined in section 2.3.9.

2.3.7 Ligation

Restriction digests of the destination vector and the vector containing insert were prepared separately using the same buffer and appropriate enzymes. The digests were then separated using agarose gel electrophoresis, as outlined in section 2.3.3, and the digested band(s) of the appropriate size was/were excised using a clean scalpel. The DNA from the gel slice(s) was/were purified as outlined in section 2.3.4. The vector and insert were mixed at a ratio of 1:3. The final reaction volume was 10 µl, and this included 1 µ of 10x T4 DNA ligase buffer and 1 µl of T4 ligase. Ligations were incubated at 16 °C overnight to allow the complimentary ends to anneal. The following morning the whole ligation mixture was used to transform DH5α cells as outlined in section 2.3.9.

2.3.8 Preparation of competent E. coli

A single colony from a freshly streaked agar plate of the appropriate strain was used to inoculate 5 ml of 2YT. This culture was grown overnight at 37 °C with shaking at 250 rpm. The following morning this culture was used to inoculate 500 ml of 2YT. The culture was grown at 37 °C with shaking at 250 rpm until the optical density (OD) at 600 nm was roughly 0.6. At this point the culture was spun down at 4000 xg for 10 minutes (in a Beckman bench top centrifuge) and resuspended in 50 ml ice cold $CaCl_2$ on ice for 30 minutes. Following this incubation the cells were again pelleted at 4000 xg and resuspended in ice cold $CaCl_2$, to which 15 % (w/v) glycerol was added. Aliquots were then frozen in liquid nitrogen and stored at -80 °C until needed.

2.3.9 Transformation of E. coli cells

Competent *E. coli* were defrosted from -80 °C for 15 minutes on ice. Plasmid was added and the cells incubated for a further 15 minutes on ice. Cells were then heat shocked for exactly 45 seconds at 42 °C in a water bath and then returned to the ice for 1 minute to recover. After this minute 250 µl of room temperature SOC media was added to each transformation. Each transformation was then incubated at 37 °C with shaking at 250 rpm

for 1 hour. *E.coli* were then plated onto appropriate antibiotic plates at different dilutions and these were incubated upside down at 37 °C overnight.

2.3.10 Small scale DNA preparations (miniprep)

Single bacterial colonies were picked from fresh selective agar plates and grown in 5 ml of 2YT in a sterile universal containing the appropriate antibiotic overnight at 37 °C with shaking at 250 rpm. Plasmid DNA was then extracted from the cell pellet using the QIAprep™ spin miniprep kit following the manufactures instructions. Briefly, 3 ml of cells were pelleted in a 1.5 ml eppendorf by spinning for 5 minutes at 14,000 xg in a microfuge. The cell pellet was thoroughly resuspended in 250 µl of Buffer P1 (containing Rnase A). Following resuspension cells were lysed by the addition of 250 µl Buffer P2, the contents of the tube being mixed thoroughly by inversion. Buffer N3, 350 µl, was then added to the tube and the contents once again mixed by inversion. Precipitated protein and lipids were then removed from the sample by centrifugation at 14,000 x g for 10 minutes in a microfuge. The supernatant from this spin was applied to a QIAprep™ spin miniprep column and this was spun for 1 minute at 14,000 xg in a microfuge. The column was then washed first by the addition of 0.5 ml of Buffer PB followed by 0.75 ml of Buffer PE with a 1 minute spin at 14,000 x g in a microfuge between each wash. In order to ensure the removal of ethanol from the column, from the wash with Buffer PE, the column was finally spun for a further 2 minutes. The DNA was then eluted in 50 µl of nuclease-free water which was pipetted into the centre of the column and incubated for 1 minute before the DNA was collected in a fresh sterile 1.5 ml eppendorf tube by centrifugation at top speed for 1 minute.

2.3.11 Large scale DNA preparations (maxiprep)

Large-scale DNA preparations were made using a QIAfilter™ plasmid maxi kit, following the high copy number plasmid protocol. A single colony was picked from a selective agar plate and used to inoculate 5ml of 2YT containing the appropriate antibiotic. This culture was grown overnight at 37 °C with shaking at 250 rpm. The following evening 1 ml of this culture was used to inoculate 50 ml of 2YT containing the appropriate antibiotic and this culture was grown overnight at 37 °C with shaking at 250 rpm. The cells from this culture were pelleted by centrifugation at 4,000 xg for 10 minutes (in a Beckman table top centrifuge) and the cell pellet was resuspended in 10 ml of Buffer P1 (containing RNase

A). The cells were lysed by the addition of 10 ml of Buffer P2 which was thoroughly mixed with the resuspended cells and then incubated at room temperature for 10 minutes. The reaction was neutralised by the addition of 10 ml of Buffer P3. The mixture was then poured into a Qiagen filter and allowed to sit for ten minutes. In the meantime a P100 Qiagen column was washed with 10 mls of Buffer QBT, which was allowed to thoroughly drain by gravity. Following the ten minute period the supernatant from the Qiagen filter was pushed through the filter into the column and this was allowed to run through the column by gravity. The column was then washed with 2 x 30 ml of Buffer QC (with ethanol). The DNA was then eluted into a 50 ml sterile tube by the addition of 15 ml of Buffer QF. Isopropanol (10.5 ml) was added to the DNA solution and mixed well. Precipitated DNA was collected by centrifugation at 15,000 xg for 30 minutes in a JA-20. The supernatant was discarded and the pellet was washed with 10 ml of 70 % (v/v) ethanol. The Tube was then recentrifuged at 15,000 xg for ten minutes in a JA-20 and the ethanol was carefully poured off and the pellet of DNA allowed to air dry for approximately 20 minutes. The air dried pellet was then resuspended in 200 µl of nuclease free water. The DNA concentration was assessed spectrophotometrically.

2.3.12 DNA sequencing

DNA sequencing was carried out by Dundee University sequencing service. This was performed after all cloning to ensure error free sequence.

2.4 Protein Expression and Purification

2.4.1 Recombinant protein production in E. coli

A single colony from a fresh selective agar plate was used to inoculate an overnight culture, containing the appropriate antibiotics. This overnight culture was typically 1/10 of the volume of the final culture volume. The overnight culture was grown at 37 °C overnight with shaking at 250 rpm. The following morning, this was used to inoculate larger cultures directly, in the case of kanamycin resistance, or in the case of ampicillin resistance spun down at 4000 xg for 10 minutes and the pellet used to inoculate a fresh culture. The ampicillin resistance gene codes for β-lactamase, which when secreted breaks down ampicillin which can lead to the loss of plasmid in some cells. The spin at 4000 xg removes any β-lactamase which may have been secreted into the culture overnight.

Typically cultures were grown at 37 °C with shaking at 250 rpm until the OD at 600 nm was roughly 0.6. At this point, protein production was induced by the addition of Isopropyl-β-D-Thiogalactopyranoside (IPTG) (0.2 – 1 mM). Protein production was typically induced for 3-4 hours at 37 °C or overnight at 22 °C, before the cells were harvested by centrifugation at 4,000 xg for 20 minutes. Cell pellets were resuspended in the appropriate buffer and either processed for purification immediately or stored at – 80 °C.

2.4.2 *Recombinant protein purification*

Proteins were purified by use of tags at either the N- or C-terminus. Two tags were utilised, Hexa-HIS (HIS) and GST. Cell pellets were resuspended in HIS-purification buffer (containing EDTA-free complete protease inhibitor tablet and 1 mM Phenylmethylsulfonyl fluoride (PMSF) or GST-purification buffer (containing complete protease inhibitor cocktail and 1 mM PMSF) depending on the tag utilised to purify that particular protein. Lysoyzme was added to the resuspended cells to a final concentration of 1 mg/ml from a 100 mg/ml stock, and the tube was inverted several times to ensure thorough mixing. The mixture was incubated for 30 minutes on a rotator in the cold room. The lysate was then sonicated 4 x 30 sec using a Sanyo Soniprep 150 sonicator, set at an amplitude of 15 microns, with a 30 sec pause between sonications. DNA was then digested by the addition of DNase I (10 µg/ml) for 30 minutes at 4 °C. The lysate was clarified by centrifugation for 1 hour at 30,000 xg in a Beckman JA-20 rotor at 4 °C. The clarified lysate was then transferred to a 50 ml centrifuge tube containing 2.5 ml bed volume of washed Ni-NTA agarose (Qiagen) for HIS-tagged proteins, or glutathione sepharose (Amersham) for GST-tagged proteins. The tube was sealed with parafilm and placed on a roller for 2 hours at 4 °C. Following centrifugation at 500 xg for 5 minutes in a benchtop centrifuge, the supernatant was removed.

NTA agarose was washed five times with 50 ml 100 mM HEPES, 200 mM KCl, 15 mM imidazole pH8. The protein was eluted by incubation for 5 minutes with 2.5 mls of 100 mM HEPES, 200 mM KCl, 250 mM imidazole pH8.0 and this was repeated three times. Eluted protein was collected by spinning the beads at 500 xg for 5 minutes and removing the supernatant.

Glutathione sepharose was washed 3 times with 50 ml of PBS pH7.4+ 1 % triton, followed by 3 times with 50 ml of PBS pH7.4/0.5 M NaCl, and subsequently three times with 50 ml of PBS pH7.4. Protein was eluted from the beads by either incubating the beads for 5 minutes with 50 mM Tris , 10 mM reduced glutathione pH8.0 or incubation with 25 Units of thrombin in PBS pH7.4 for 4 hours at room temperature to cleave the fusion protein for the GST tag.

The concentration of protein in each eluted fraction was analysed as outlined in section 2.4.3.

2.4.3 Protein estimation

Protein concentrations of purified protein fractions was measured using Bio-Rad Bradford protein assay reagent. Bovine serum albumin (1 – 20 µg) was used as the standard. The amount of sample assayed was decided on so the absorbance would fall within the linear range of the standard. Assays were set up in duplicate, in 1 cm pathlength disposable cuvettes, according to the manufactures instructions and absorbance measured at 595 nm. Protein concentrations were calculated using a curve derived from the standard values.

2.4.4 GST pull-downs

Protein, 5 µg of GST or GST-tagged protein, was incubated with 10 µl of glutathione sepharose in a volume of 100 µl of binding buffer (150 mM Potassium acetate, 1 mM $MgCl_2$, 0.05 % Tween 20, 20 mM Hepes pH 7.4) for 1 hour in the cold room with end-over-end rotation. Unbound protein was removed by washing the beads with three 0.5 ml washes of binding buffer, beads were collected by centrifugation at 500 xg in a microfuge at 4 °C for 2 minutes. HIS tagged protein was added (0 – 50 µg) to appropriate tubes in a total volume of 500 µl of binding buffer and this was incubated with the beads overnight in the cold room with end-over-end rotation. Unbound protein was removed by washing three times with 1 ml of binding buffer with the addition of 1 mg/ml fish skin gelatine, followed by three washes with 1 ml of binding buffer with 5 % (w/v) glycerol and three washes with 1 ml of binding buffer alone. Between each wash the beads were pelleted by centrifugation at 500 xg in a microfuge at 4 °C for 2 minutes. After the final wash the beads were pelleted by centrifugation at 500 xg in a microfuge at 4 °C for 2 minutes and all remaining supernatant carefully removed. The beads were then resuspended in 15 µl of 1X

SDS-PAGE sample buffer. The samples were boiled for 5 minutes and eluted protein was analysed by running on a 12 % SDS-PAGE gels as outlined in section 2.2.1 followed by staining in Coomassie blue as outlined in section 2.2.4. A similar amount of GST bound protein was present in each experiment.

2.5 Fusion assay

2.5.1 Full-length SNARE expression and purification

To purify the syntaxin 4 / SNAP23 complex plasmids encoding both of these proteins were both transformed at the same time into BL21 DE3 cells. Cells containing both plasmids were selected for on dual antibiotic plates. A single colony was used to inoculate an overnight starter culture, 1/10 of the volume of the induction culture containing 500 µg/ml ampicillin and 50 µg/ml kanamycin. The following morning the overnight culture was spun down at 4,000 xg for 10 minutes, the supernatant removed, and the pellet used to inoculate 1 litre cultures containing 200 µg ampicillin and 25 µg kanamycin, which were grown at 37 °C with shaking at 250 rpm. An additional 100 µg ampicillin was added each hour. These 1 litre cultures were grown until the OD at 600 nm was roughly 0.6. At this point protein production was induced by the addition of 1 mM IPTG. Protein production was induced overnight at 25 °C with shaking at 250 rpm.

The cells were harvested the next morning by centrifugation at 4,000 xg for 30 minutes. The cell pellet was then resuspended in A200 containing complete protease inhibitors and 2 mM PMSF. Following resuspension, ¼ volume 20 % Triton X100 was added to the resuspended cells. The cells were broken by two passes through a French press at 950 psi. Insoluble matter was removed by centrifugation at 30,000 xg for 1 hour in a JA-20 rotor. The protein was then purified from the supernatant was using glutathione sepharose. Protein in the supernatant was incubated with 5 mls of pre-equilibrated glutahtione sephaorse overnight at 4 °C. The following day, the beads were pelleted by spinning at 500 xg for 5 minutes, the supernatant was removed and the beads resuspended in 20 ml of A200 containing 1 % Triton X100, and applied to a Bio-Rad Eco-pac disposable chromatography column. The buffer was allowed to flow through, and the column was washed with 5 column volumes (20mls) of A200 contaning 1 % Triton X100. The triton was then exchanged for n-octyl-β-d-glucopyranoisde (OG) by washing the column 10 times with 15 mls of A200 + 1 % OG. The bottom of the column was sealed using the cap

provided and nescofilm. The beads were resuspended in an equal volume of A200 containing 1% OG, and 125 Units of thrombin were added. The top of the column was sealed with the provided cap and nescofilm. The beads were incubated on a rotator for 4 hours at room temperature, following which the top and the bottom of the column were uncapped and the supernatant was collected into a clean 15 ml corning tube from the bottom of the column by gravity. The eluate was then aliquoted into 550 µl aliquots, snap frozen in liquid nitrogen and stored at –80 °C until use.

VAMP2 was expressed in a similar manner with the exception of addition of kanamycin to the culture medium. The cultures were grown to an OD at 600 nm of roughly 0.8. Protein production was then induced by the addition of 1mM IPTG for 3 hours at 37 °C. Cells were resuspended, broken and cell lysate centrifuged as outlined for syntaxin 4 / SNAP23. The supernatant was then purified using Ni-NTA agarose. Pre-equilibrated Ni-NTA agarose, 3 ml bed volume, was incubated with the supernatant for 2 hours. The beads were then washed once with 50 mls of A200 containing 1 % Triton X100, and the beads were collected by centrifugation at 500 xg for 5 minutes. The beads were resuspended in 20 ml of A200 containing 1 % Triton X100 and applied to a Bio-Rad Eco-pac disposable chromatography column. The buffer was allowed to run through by gravity and the beads were washed with 5 column volumes (20 mls) of A200 containing 1 % triton. The Triton X100 was then exchanged for OG by washing the column 10 times with 10 mls of A200 containing 1 % OG and 20 mM imidazole. The protein was then eluted from the column by incubating the beads with 3 mls of A200 containing 1 % OG and 500 mM imidazole for 30 minutes in a sealed column, following which the top and the bottom of the column were uncapped and the supernatant was collected into a clean 15 ml corning tube from the bottom of the column by gravity. Thrombin was inhibted by the addition of 2 mM AEBSF. The eluate was then aliquoted into 550 µl aliquots, snap frozen in liquid nitrogen and stored at –80 °C until use.

2.5.2 *Lipid stocks*

Lipid stocks were made up in chloroform and stored at – 80 °C under nitrogen. For t-SNARE liposomes a 15 mM lipid stock was made up in chloroform containing 85 mol% POPC and 15 mol% DOPS. For v-SNARE liposomes a 3 mM lipid stock was made up in chloroform containing 82 mol% POPC, 15 mol% DOPS, 1.5 mol% NBD-DPPE and 1.5

mol% rhodamine-DPPE. Trace amounts of [^{3}H]-DPPC (1,2-dipalmitoyl phosphatidylcholine) are added to monitor lipid recovery.

2.5.3 Lipid resuspension, detergent dilution and dialysis

100 µl of 15mM unlabelled lipid stock, for t-SNARE liposomes, or 500 µl of 3 mM labelled lipid stock, for v-SNARE liposomes was placed at the bottom of a 12 x 75 mm glass test tube. The chloroform was then evaporated off using a stream of nitrogen for 15 minutes in a fume hood. To ensure that the lipid films were completely dry the samples were then dried for 30 minutes under vacuum. Purified t- or v-SNARE (500 µl) purified as outlined in section 2.5.1, containing 1 % OG, was then added to each tube. The lipid film was then completely resuspended, by vortexing for 15 minutes.

After the lipid film was completely resuspended the detergent was diluted below its critical micellar level by the addition of 1 ml of Buffer A200 + 1 mM DTT. This was added dropwise while the sample was continuously vortexed.

After the detergent had been diluted the samples were dialysed to remove any remaining detergent. The samples were placed into 3 ml Float-a-Lyzers with a MWCO of 10,000 pre-equilibrated in dialysis buffer and these were floated in 4 litres of Buffer A200 containing 1 mM DTT with the addition of 4 g of Bio-Beads, with stirring, in the cold room overnight. Samples were recovered the following day, and placed at the bottom of a SW60 tube on ice for subsequent separation using gradient centrifugation.

2.5.4 Proteoliposome recovery

Proteoliposomes were recovered by floatation on a nycodenz gradient. All solutions were pre-chilled prior to use. An equal volume of 80 % nycodenz in buffer A200 containing 1 mM DTT was mixed with the recovered dialysate to produce a 40 % nycodenz mixture. This was overlaid with 1.5 ml of 30 % nycodenz in buffer A200 containing 1 mM DTT. This layer was then overlaid with 250 µl of glycerol free A200. These gradients were then spun for 4 hours at 46,000 rpm in an SW60 rotor at 4 °C. Proteoliposomes float to the interface between the glycerol free A200 and the 30 % nycodenz, due to their lipid content, and free protein remains in the 40 % layer. Proteoliposomes were recovered from the top

of the gradient by removal of 400 µl in a 1.5 ml eppendorf, snap frozen in liquid nitrogen and stored at – 80 °C.

2.5.5 *Proteoliposome characterisation*

Proteoliposomes were characterised by Transmission Electron Microscopy. Briefly, a carbon coated quantifoil was glow discharged to increase the hydrophobicity of the carbon surface. The quantifoil was then placed carbon face down on a 10 µl drop of liposome on nescofilm. The Quantifoil was then put through five 10 µl drops of dH_20 to remove buffer, for 10 seconds per drop. The liposomes were stained by incubating the quantifoil face down on a 10 µl drop of carbohydrate/negative stain mixture of 5% w/v ammonium molybdate for 5 seconds. The quantifoil was secured in forceps and excess negative stain wicked away using filter paper touched to the edge of the grid. The negative stain was allowed to dry for 10 minutes prior to analysis by Transmission Electron Microscopy.

2.5.6 *Fusion assays*

Typically fusion assays were set up by mixing 5 µl of v-SNARE liposome with 45 µl of t-SNARE liposome directly in a well of a 96 well microtitre plate, on ice. This was then sealed using sticky tape and incubated overnight at 4 °C. For assays requiring the addition of soluble v-SNARE, 2 µl of purified protein in buffer A200 was added to the t-SNARE liposomes on ice for 10 minutes prior to the addition of v-SNARE liposomes. To correct for the resulting difference in volume 2µl of A200 was added to all other wells in that run.

The microtitre plate was rapidly warmed to 37 °C for 20 seconds by floating in a 37 °C water bath, and then fluorescence was monitored in a fluorescent plate reader. The fluorescence was measured for 2 hours with the excitation set to 460 nm and the emission recorded at 538 nm at 2 minute intervals. After this period, the plate was removed and 10 µl of 2.5 % (w/v) n-dodecylmaltoside was added to each well. The plate was gently mixed for 2 minutes, and then fluorescence was recorded, as before, for 40 minutes at 2 minute intervals.

2.5.7 Data analysis

Raw fluorescence data was transferred to KaleidaGraph, Synergy Software, a graphing and analysis programme. Raw fluorescence was plotted against time. This was then normalised to percentage maximal detergent signal and percentage maximal detergent signal was plotted against time. Using an equation derived from a calibration curve percentage maximal detergent signal was converted to fold lipid dilution or "rounds of fusion" and this was plotted against time (see section 3.2.5)

2.6 Cells and culture conditions

2.6.1 Passage of Sf9 cells

Sf9 cells were grown in 150 cm^2 flasks containing Sf900-II with 1 % (v/v) penicillin and streptomycin. Cells were cultured at 27 °C in a humidified atmosphere with no CO_2. The media was replaced every 48 hours.

Sf9 cells were split when they reached roughly 90% confluency. Following aspiration of the media from a 150 cm^2 flask, fresh media was added and the cells monolayer was dislodged by rapping the flask numerous times. This cell suspension was then added to 5 times the original culture volume of fresh media, and split between the desired number of plates or flasks.

2.6.2 Freezing down cells

Sf9 cells were frozen down in 50 % conditioned medium, in which cells had been grown for 2 days, and 50 % fresh medium containing 10 % DMSO, which was filter sterilised and pre-chilled to 4 °C. Cells, which had been grown to roughly 80 % confluency, were dislodged by rapping the flask numerous times. At this point cells were counted. The cell suspension was then transferred to a sterile falcon tube, and centrifuged for 5 minutes at 500 xg. The supernatant was aspirated, and the pellet was resuspended gently in the appropriate volume of chilled, freezing-down media to give a cell density of more than 1 x 10^7 cells/ml. Once thoroughly resuspended, the suspension was transferred to a cryo-vial and placed at –80 °C overnight, insulated in blue roll and a polystyrene box, to freeze slowly, before being transferred to a liquid nitrogen vat.

2.6.3 Resurrection of frozen cell stocks from liquid nitrogen

A vial of frozen cells, prepared as outlined in section 2.6.2, was removed from liquid nitrogen and transferred immediately to a 37 °C water bath. The cells were thawed with occasional agitation, and pipetted into a 75 cm^2 flask containing SF900-II, 1 % (v/v) penicillin and streptomycin which had previously been equilibrated at 27 °C in a humidified atmosphere. The cells were allowed to attach to the flask for 1 hour. Following this, the media was aspirated from the flask, and replaced with 15 ml of fresh media. Once the cells reached 90 % confluency, they were split appropriately, as outlined in section 2.6.1.

2.7 Baculovirus Production

2.7.1 Homologous recombination

For each transformation a vial of DH10 Bac™ cells was thawed on ice and 100 µl of cells were transferred to a sterile 15 ml corning tube. To each tube 1ng of pFastBac™ Dual DNA was added and the cells were then incubated on ice for 30 minutes. The cells were heat shocked in a water bath prewarmed to 42 °C, without shaking, for 45 seconds and allowed to recover on ice for two minutes. After recovery 900µl of room temperature SOC was added to each tube and the cells were incubated at 37 °C for 4 hours with shaking at 225 rpm. During this incubation LB selective plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracylcin, 40 µg/ml IPTG and 300 µg/ml Bluo-Gal using the stock solutions indicated below, were poured and prewarmed to 37 °C prior to the plating of cells.

Component	**Stock soln.**	**Final Conc.**
Kanamycin	10 mg/ml (in distilled water)	50 µg/ml
Gentamicin	10 mg/ml (in distilled water)	7 µg/ml
Tetracycline	5 mg/ml (in ethanol)	10 µg/ml
IPTG	200 mg/ml (in distilled water)	40 µg/ml
Bluo-Gal	20 mg/ml (in DMSO)	300 µg/ml

A portion of the neat transformation, 100 µl, as well as 1:10 and 1:100 dilutions were plated on the selective plates, which were incubated at 37 °C for 48 hours. Following this incubation colonies were restreaked on selective plates and incubated for a further 24 hours at 37 °C to verify the white phenotype. From these plates single white colonies were selected and used to inoculate a 2 ml culture of 2YT containing 50 µg/ml Kanamycin, 7 µg/ml gentamicin and 10 µg/ml tetracycline. These cultures were grown for 48 hours at 37 °C with shaking at 225 rpm.

2.7.2 Purification of recombinant DNA

Precipitation was carried out as outline in the Bac-to-Bac manual with modifications. Briefly a portion of the bacterial suspension from the 2YT culture, 1.5 ml, was spun down in a 1.5 ml eppendorf at 14,000 xg for 1 minute to pellet the cells. The remaining bacterial suspension was used to make glycerol stocks which were stored at - 80 °C. The pellet was thoroughly resuspended in 0.3 ml of Buffer P1 (from a Qiagen miniprep kit). The resuspended pellet was then mixed with 0.3 ml of buffer P2 (from a Qiagen miniprep kit) and this was incubated for 5 minutes at room temperature. Following this incubation 0.3 ml of 3M Potassium acetate pH 5.5 was slowly added to the tube and the mixture was placed on ice for 10 minutes. The sample was then centrifuged at 14,000 xg for 10 minutes to remove genomic DNA and precipitated protein. The supernatant from this spin was transferred to a clean 1.5 ml tube containing 0.8 ml of isopropanol and mixed thoroughly by inversion before being placed on ice for 10 minutes. The samples were then centrifuged for 5 minutes at 14,000 xg at room temperature. The supernatant was removed and the pellet was air-dried. The dried pellet was then resuspended in 40 µl of sterile TE buffer pH 8.0 with gentle agitation. The resuspended DNA was stored at 4 °C.

2.7.3 Production of baculovirus particles in Sf9 cells

Briefly, Sf9 cells (in log phase) were seeded into each well of a 6 well plate in 2 ml of Sf900-II, containing antibiotics, at a density of 9 x 10^5/well and allowed to attach for an hour. During attachment the transfection mixture was made up. For each transformation 5 µl of bacmid DNA was added to 100 µl of unsupplemented Graces insect medium. The transfection reagent cellfectin, 6 µl, was diluted with a further 100 µl of unsupplemented Graces insect medium. The two were subsequently mixed and incubated at room temperature for 30 minutes. Each well containing insect cells was washed once with 2 ml

of unsupplemented Graces insect medium. Each transfection mixture was made upto 1ml with unsupplemented Graces insect medium and then added drop-wise to the relevant wells. The insect cells were incubated with the transfection mixture for 5 hours at 27 °C. After this incubation the DNA:lipid mix was removed and replaced with 2 ml of Sf900-II containing antibiotics. The cells were incubated for 72 hours at 27 °C in order to produce the P1 recombinant virus.

After the 72 hour incubation the supernatant from each well, which should contain the virus, was removed and transferred into a sterile 15 ml corning tube. The supernatant was spun at 500 xg for 5 minutes in order to clear any cell debris. The supernatant was transferred into a sterile 50 ml corning tube and filtered through a 2 μM low protein-binding filter into a new sterile 15 ml corning tube. Baculovirus stocks were stored in the dark at 4 °C.

2.8 Analysis of Palmitoylation

Lysate from Sf9 cells infected with recombinant baculovirus for 72 hours was prepared in lysis buffer (A200, 1 %OG, 2 mM PMSF and complete protease inhibitors) containing 25 mM NEM. The cells were broken by ten passes through a 10 gauge needle. The protein content was assayed as outlined in section 2.4.3, and 100 μg of protein in a total volume of 700 μl was placed in two separate eppendorf tubes. The lysate was incubated at 4 °C for 10 minutes. Following this incubation, protein was methanol-chloroform precipitated. To each tube 150 μl of methanol and 600 μl of chloroform was added. The tubes were vortexed thoroughly to mix and spun at 14,000 xg in a microfuge. The upper layer was gently removed and a further 450 μl of methanol added. The tubes were again vortexed and protein was pelleted by centrifugation at 14,000 xg in a microfuge. The protein pellet was allowed to airdry. This pellet was resuspended in 50 μl of high urea buffer (2 % SDS, 8 M Urea, 100 mM NaCl, 50 mM Tris pH 7.4) by sonication for 30 minutes in a sonicating water bath. To one tube 700 μl of 1M hydroxylamine pH 7.4 was added and to the other 700 μl of 1M Tris pH 7.4. To each tube 300 μM biotin-BMCC (from Pierce) was added. The tubes were then incubated in the cold room for 2 hours with end-over-end rotation. Protein was then methanol-chloroform precipitated and resuspended in high urea buffer as described above. The resuspended protein was diluted in 2 ml of lysis buffer and incubated with 100 μl of streptavidin-agarose (pierce) for 1 hour at room temperature with end over end rotation. Beads were thoroughly washed 3 times with 1 ml of PBS

containing 0.5 M NaCl and 0.1 % Triton X100 and collected by centrifugation at 14,000 xg in a microfuge. The beads were finally washed with 1 ml of PBS, collected by centrifugation in a microfuge at 14,000 xg and resuspended in 20 µl of high urea buffer and 40 µl of 4 X SDS-PAGE sample buffer. The beads were boiled for 5 minutes and eluted protein was run on a 10 % SDS-PAGE gel as outlined in section 2.2.1, transferred to nitrocellulose as outlined in section 2.2.2, and probed for GST-SNAP23 using an antibody raised against SNAP23, as outlined in section 2.2.3.

Chapter 3

In vitro Fusion Assay using the SNAREs involved in Glut4 vesicle exocytosis, syntaxin 4, SNAP23 and VAMP2

3.1 Introduction

Since the discovery of SNARE proteins in neuronal tissue in 1993 (Sollner *et al.*, 1993) many homologues of these proteins have been identified (Jahn and Scheller 2006). Each vesicle fusion event within the cell is now known to involve members of this highly conserved family of proteins (Jahn and Scheller 2006). Several studies have shown that the plasma membrane t-SNARE complex, syntaxin 4 and SNAP23, and the v-SNARE present on Glut4 containing vesicles VAMP2 mediate the insulin-stimulated fusion of Glut4 containing vesicles to the plasma membrane of insulin-responsive cells as outlined in section 1.6.

3.1.1 Syntaxin 4

Syntaxin 4, a member of the syntaxin family, encodes a 298 amino acid 34 kDa protein expressed in a variety of cells types (Bennett *et al.*, 1993). Syntaxin 4 is predicted to contain four domains, a C-terminal transmembrane tail (Teng *et al.*, 2001), a SNARE domain through which interactions with other SNAREs are mediated, and a N-terminal domain composed of 3 α-helices which have been predicted to form a three helical bundle (Fernandez *et al.*, 1998). There is strong evidence supporting the role of syntaxin 4 in insulin-stimulated Glut4 vesicle fusion. This isoform of syntaxin has been shown to be predominantly expressed at the cell surface of adipocytes (Volchuk *et al.*, 1996;Tellam *et al.*, 1997), and studies to perturb its actions have been shown to substantially inhibit insulin stimulated Glut4 translocation (Cheatham *et al.*, 1996;Olson *et al.*, 1997) and insulin-stimulated glucose transport (Volchuk *et al.*, 1996;Tellam *et al.*, 1997).

3.1.2 SNAP23

SNAP23 is a ubiquitously expressed protein of 210 amino acids with a predicted molecular weight of 23 kDa (Ravichandran *et al.*, 1996). SNAP23 is highly homologous to SNAP25 (Wang *et al.*, 1997), and is predicted, like SNAP25, to be made up of two SNARE domains connected by a linker domain that is palmitoylated in a cysteine rich region thought to mediate attachment to the membrane (Hodel, 1998). SNAP23 was isolated from adipocytes by virtue of its ability to bind the cytoplasmic domain of syntaxin 4 in a yeast two-hybrid screen (Ravichandran *et al.*, 1996). Similar to syntaxin 4 it is predominantly localised to the plasma membrane of adipocytes (Wang *et al.*, 1997). The perturbation of

the function of SNAP23, like that of syntaxin 4, also led to a substantial inhibition of Glut4 translocation to the plasma membrane (Rea *et al.*, 1998;Foster *et al.*, 1999;Kawanishi *et al.*, 2000).

3.1.3 VAMP2

VAMP2 encodes a polypeptide of 116 amino acids yielding a protein of 16 kDa. This v-SNARE is enriched on Glut4-containing vesicles (Volchuk *et al.*, 1995). The perturbation of the function of VAMP2 by the introduction of the soluble cytoplasmic domain of VAMP2 into adipocytes inhibited insulin-stimulated Glut4 translocation (Cheatham *et al.*, 1996;Martin *et al.*, 1998;Millar *et al.*, 1999), strongly supporting its role as the v-SNARE in the exocytosis of Glut4 containing vesicles.

3.1.4 Syntaxin 4, SNAP23, VAMP2 ternary complex

It has been demonstrated that *in vitro* VAMP2, SNAP23 and syntaxin 4 form an extremely stable SDS-resistant SNARE complex much like that of the neuronal exocytic SNARE complex (Rea *et al.*, 1998).

The studies undertaken so far strongly suggest that VAMP2, v-SNARE present on insulin responsive Glut4 storage vesicles interacts with the binary complex formed by the binding of syntaxin 4 to SNAP23 present on the plasma membrane to facilitate exocytosis of Glut4 storage vesicles. Although it has been assumed that these SNAREs come together to form a complex capable of supporting fusion this is yet to be definitively shown. There is also little known about the regulation of complex formation by these SNAREs.

3.1.5 In vitro fusion assay for membrane fusion

In vitro fusion assays have used either partially intact purified cellular components or completely artificial liposomes reconstituted with recombinant proteins (reviewed in (Avery *et al.*, 1999). These types of assays allow the study of protein-mediated events in a defined setting, and allow the addition or depletion of various factors to study the effect these have on the fusion process.

The most widely adapted *in vitro* fusion assay for SNARE mediated fusion is based on a fluorescence energy resonance transfer (FRET) lipid mixing assay described in 1980 by

Struck and colleagues (Struck *et al.*, 1981). This assay makes use of a FRET pair of fluorophores, NBD (N-(7-nitro-2,1,3-benzoxadiazol-4-yl)) and Rhodamine (N-(lissamine rhodamine B sulfonyl)), covalently attached to lipid species in the reconstituted liposomes. The assay utilises the overlap in the excitation and emission spectrum of this pair. When the molecules are within close proximity, the excitation of NBD leads to emission, which is absorbed by Rhodamine. This transfer is extremely dependent on the proximity of the fluorophores. When the liposomes fuse, as shown in Figure 3.1, the bulk lipid in the resulting liposome is diluted by a factor of 2, and fusion can be measured as an increase in NBD fluorescence at 535 nm. Using an assay, based on this original assay it has been shown that the exocytic SNARE complex, formed by the t-SNARE complex of syntaxin 1a/SNAP25 and the v-SNARE VAMP2, is sufficient and necessary to promote membrane fusion of liposomes (Weber *et al.*, 1998), although the fusion is much slower than that observed physiologically (Wolfel and Schneggenburger, 2003).

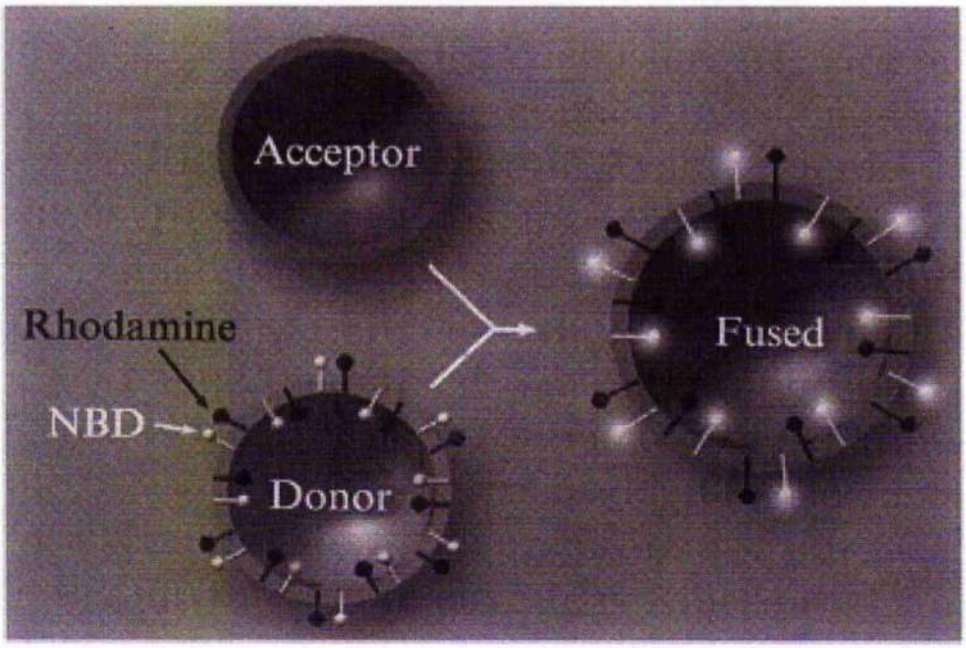


Figure 3.1: The principle of the in vitro fusion assay

The liposome fusion assay makes use of two head group labelled lipids, one labelled with NBD (light spheres) and one with Rhodamine (dark spheres). A quenched mixture of these lipids is reconstituted in the same membrane (v-SNARE containing liposomes, Donor). When fusion occurs between labelled liposomes and unlabelled liposomes (t-SNARE containing liposomes, Acceptor) the group labelled lipids are diluted and this can be measured as an increase in NBD emission with time (Taken from Scott *et al.*, 2003).

3.2 Aims of this chapter

The aims of this chapter were to purify the three full-length SNAREs: syntaxin 4, SNAP23 and VAMP2, and reconstitute them into synthetic liposomes in order to establish an *in vitro* system to study the regulation of fusion facilitated by this SNARE complex.

In this chapter I have successfully purified all three SNAREs, reconstituted them into liposomes, and successfully performed fusion assays using these reconstituted liposomes.

3.3 Results

In order to establish a fusion assay using these SNARE proteins, we first had to express and purify the full-length versions of these proteins. Recombinant DNA technology allows the addition of tags to proteins that can be extremely useful, when used with chromatography procedures, enabling the purification of proteins to high purity and high yield. The SNARE proteins successfully reconstituted thus far in the *in vitro* fusion assay have all been expressed and purified from *E. coli* as tagged proteins. Since most SNARE proteins have a C-terminal transmembrane tail the successful purification of these proteins from *E. coli* relies on the inclusion of detergent throughout the purification process.

3.3.1 Expression and purification of cytoplasmic and full-length VAMP2.

We chose to express full-length VAMP2 as a Hexa-HIS-tagged protein in BL21 DE3 cells, and to purify this protein using Ni-NTA agarose chromatography. VAMP2 was expressed as a C-terminally HIS tagged protein from the plasmid pET-VAMP2FL-myc-HIS which was a kind gift from Giampietro Schiavo (London, UK). A fraction from each of the steps involved in purification was analysed by SDS-PAGE followed by Coomassie staining, as shown in Figure 3.2A. It was found that this protein was expressed at high levels in these cells and was mainly soluble in detergent.

As a control in the *in vitro* fusion assay, to ensure any fusion observed is protein mediated, the soluble cytoplasmic domain of VAMP2 is added (Weber *et al.*, 1998). This should bind to the t-SNARE complex present on the acceptor liposomes and preclude binding of VAMP2 present on the donor liposomes thus blocking fusion. Soluble VAMP2 (VAMP2 missing the C-terminal transmembrane domain) was also expressed as a HIS-tagged protein in BL21 DE3 cells and purified using Ni-NTA agarose chromatography in a similar manner to the purification of full-length VAMP2 with the omission of any detergent. The eluted fractions with the highest yield of protein were pulled and dialysed overnight against glycerol free A200, the buffer the fusion assay is performed in. A SDS-PAGE gel of the dialysed fraction is shown in Figure 3.2B.

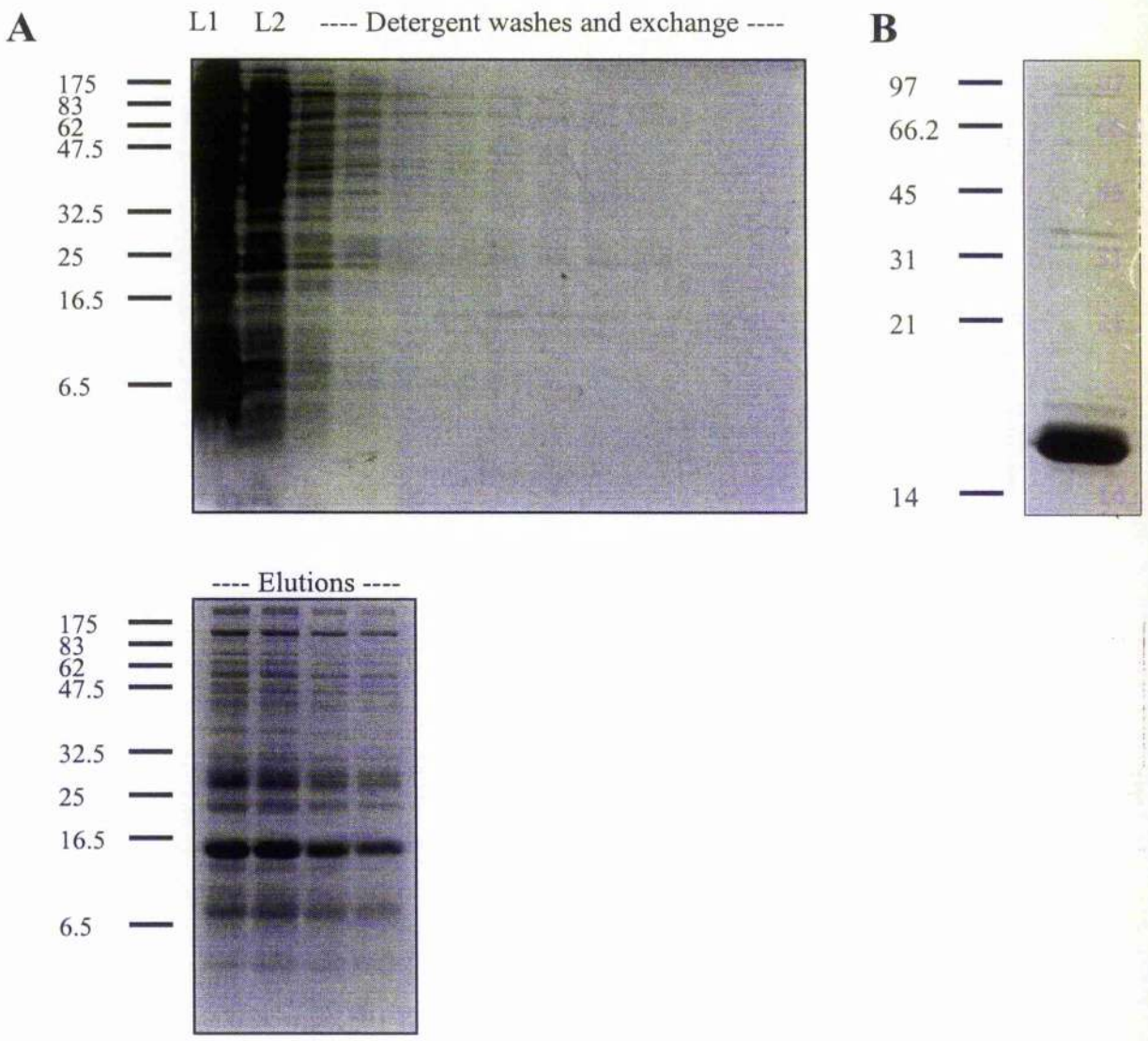


Figure 3.2: Purification and purity of full-length and the cytoplasmic domain of VAMP2

(A) Expression of full-length HIS-tagged VAMP2, in BL21 DE3 *E. coli* cells, was induced for 3 hours at 37 °C by adding IPTG and HIS-tagged VAMP2 was purified as outline in section 2.5.1. The Figure shows the purity of the pre-induction lysate (L1), post-induction lysate (L2), detergent washes, detergent exchange and fractions eluted using imidazole (200-500 mM) run on a 15 % SDS-PAGE gel and stained with Coomassie blue as outlined in sections 2.2.1 and 2.2.4. (B) The cytoplasmic domain of VAMP2 was purified in a similar manner with the omission of detergent in the purification buffers, dialysed against A200 with no glycerol and analysed for purity by SDS-PAGE on a 15 % SDS-PAGE gel followed by staining with Coomassie blue. Positions of the pre-stained broad range and Bio-rad low molecular weight protein markers are respectively indicated

As can be seen in Figure 3.2A the elutions of full-length VAMP2 contained only one major product of the correct size for full-length VAMP2, 16 kDa, indicating that the protein had been successfully expressed and purified. Likewise for the soluble cytoplasmic domain of VAMP2 only one major product was observed on SDS-PAGE analysis.

Having successfully purified the full-length and cytoplasmic domain of VAMP2 we went on to attempt to purify the t-SNARE complex of syntaxin 4/SNAP23.

3.3.2 Optimisation of expression of full-length syntaxin 4 complexed with SNAP23.

We chose to express syntaxin 4 and SNAP23 together in the same *E. coli* cell and purify them as a complex. This methodology had previously been successfully employed to express and purify the homologous neuronal t-SNARE complex consisting of syntaxin 1A and SNAP25 (Weber *et al.*, 1998). Syntaxin 4 was expressed as a HIS-tagged protein from the vector pQE30 and SNAP23 was expressed as a GST-tagged protein from the vector pET41a. These vectors contain cassettes for resistance to two different antibiotics, ampicillin for pQE30 and kanamycin for pET41a, allowing for dual selection of a single *E.coli* colony containing both vectors. Purification was carried out using glutathione sepharose, which binds specifically to the GST tag present on SNAP23. The t-SNARE complex was subsequently eluted from the glutathione sepharose by cleaving the GST tag from SNAP23 using thrombin.

Initially we attempted to express full-length syntaxin 4 and SNAP23 using similar conditions as described for VAMP2, however, this proved problematic with SNAP23 being grossly over purified compared to syntaxin 4 (data not shown). This appeared to be due to syntaxin 4 being mostly insoluble when over-expressed in *E. coli*. Further purification steps were employed using the HIS tag on syntaxin 4 and a similar purification protocol as employed for full-length VAMP2 in order to increase the concentration of syntaxin 4. While this did increase the concentration of syntaxin 4, it was still not equimolar to SNAP23. Since this recombinant SNAP23 has no palmitate groups it should not be incorporated into subsequent liposome populations. In agreement with this only the complex appeared to be reconstituted in vesicles (performed by Kirilee Wilson, data not shown). However, when a fusion assay was set up using these liposomes it was found that

the concentration of t-SNARE complex in these liposomes was too low to support fusion in this system (performed by Kirilee Wilson, data not shown).

Although *E. coli* is very useful for expressing recombinant proteins, the over expression of heterologous gene products can lead to aggregation and accumulation into insoluble inclusion bodies. The expression of many recombinant proteins has proved problematic in *E. coli,* and successful expression and purification is often due to trial and error. Many strategies have been employed for increasing solubility in *E. coli* including low IPTG concentrations, low temperatures and the co-expression of chaperone proteins (reviewed in Lilie *et al.*, 1998). Due to the poor solubility of syntaxin 4 it was necessary to optimise expression conditions to enable the purification of sufficient quantities of syntaxin 4 for the *in vitro* fusion assay. Eventually it was found that inducing protein production at an OD600 of 0.6, overnight at a low temperature (25 °C) significantly increased the solubility of syntaxin 4 (data not shown). Low temperatures have been proposed to increase protein solubility because while folding is relatively unaffected by low temperatures the rate of transcription/translation is reduced giving recombinant proteins more time to fold correctly within the *E. coli* cell.

3.3.3 Purification of full-length syntaxin 4 complexed to SNAP23

Syntaxin 4 in pQE30 and SNAP23 in pET41a were co-transformed into BL21 DE3 cells, and selected for on agar plates containing both ampicillin and kanamycin. A single colony was used to start an overnight culture and this was used to inoculate 8 litres of culture, the following morning, as outlined in section 2.5.1. Protein production was induced when the OD at 600 nm was roughly 0.6 by the addition of 1 mM IPTG for 16 hours at 25 °C. Protein was purified, as outlined in section 2.5.1, and a fraction from each step was analysed by SDS-PAGE, followed by Coomassie staining, as shown in Figure 3.3.

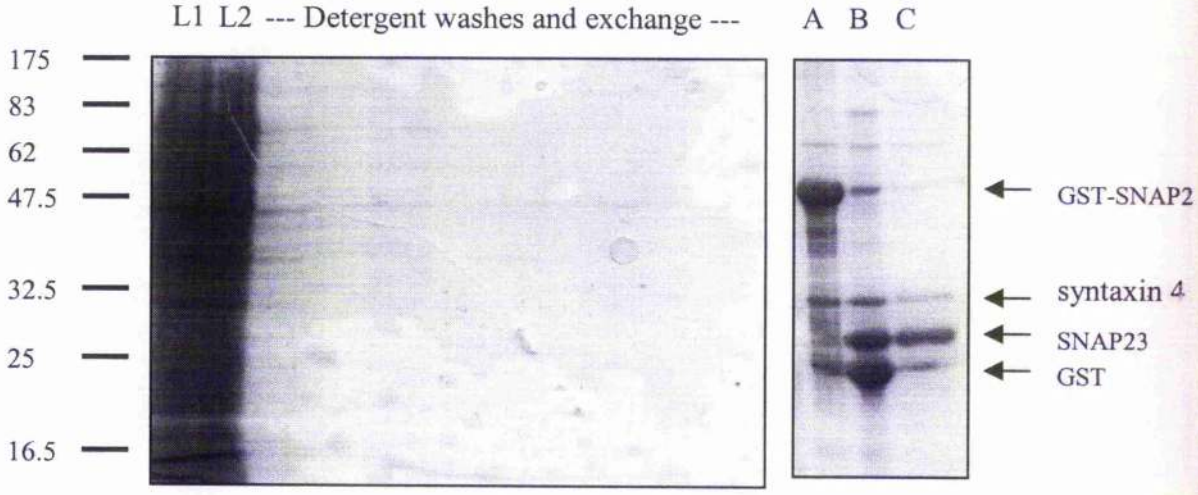


Figure 3.3: Purification and purity of SNAP23/syntaxin 4 t-SNARE complex recovered from E. coli lysates.

Full-length syntaxin 4 and SNAP23-GST were expressed in BL21 DE3 *E. coli* cells. Expression of the t-SNARE complex was induced overnight (roughly 16 hours) by adding 1 mM IPTG and the complex was purified by virtue of the GST tag on SNAP23 as outlined in section 2.5.1. The GST tag was cleaved from SNAP23 by incubation with thrombin for 4 hours at room temperature. The purity of the protein was assessed by SDS-PAGE on a 12 % SDS-PAGE gel followed by Coomassie Blue staining of the gel as outlined in sections 2.2.1 and 2.2.4. The Figure shows the purity of the pre-induction lysate (L1), post induction lysate (L2), detergent washes and exchange. The three final lanes show precleaved complex bound to glutathione sepharose (4 µl, A), bead slurry after 4 hours thrombin cleavage (4 µl, B) and recovered cleaved complex (1 µl, C). Positions of the broad range molecular weight markers are shown.

In Figure 3.3, lane A a band of the appropriate size for syntaxin 4, 34 kDa, can be seen. A further, more intense band, can be seen just above the 47.5 kDa marker, roughly the expected size for SNAP23-GST fusion. In Lane B it can be seen that this upper band is significant reduced while two new bands appear one at the 25 kDa marker, the correct size for GST, and one slightly above this which is SNAP23. The major band in the cleaved supernatant, lane C, is SNAP23 following cleavage from GST, this runs slightly higher than expected due to vector sequence that runs after the thrombin cleavage site into the coding sequence for SNAP23. The band above this band is syntaxin 4.

The protein concentration of the cleaved supernatant was estimated using the Bio-Rad protein assay as outlined in section 2.3.3. The concentration was typically estimated at roughly 1 mg/ml. The yield of syntaxin 4 was vastly improved by the change in expression conditions. Although the levels of SNAP23 and syntaxin 4 were still not equimolar, this did not pose a problem to the use of this purified protein for reconstitution into liposomes, because as mentioned previously, recombinant SNAP23 from *E. coli* cells lacks the addition of palmitate groups and therefore only SNAP23 complexed to syntaxin 4 is incoroporated into reconstituted vesicles.

Since we now had all the full-length SNAREs purified to an appropriate concentration it was possible to reconstitute these into synthetic liposomes.

3.3.4 Reconstitution of full-length recombinant SNAREs into liposomes

Lipids, labelled for VAMP2 liposomes, and unlabelled for syntaxin 4/SNAP23 liposomes, were dried to a film and re-suspended in a solution of the relevant protein in detergent as outlined in section 2.5.3. In order to reduce the concentration of OG below its critical micellar concentration, 1 ml of buffer A200, without detergent, was added as outlined in section 2.4.4. Detergent was then completely removed by dialysis overnight against buffer A200 as outlined in section 2.5.3. The following morning the liposomes were separated from soluble protein by floatation on a density gradient of nycodenz. The top fraction of this gradient, containing the liposomes, was removed and flash frozen in liquid nitrogen as outlined in section 2.5.4. Recovery of liposomes, and incorporated protein, following floatation on the nycodenz gradient was assessed by analysing 10 µl of liposomes on SDS-PAGE followed by Coomassie blue staining as outlined in sections 2.2.1 and 2.2.4.

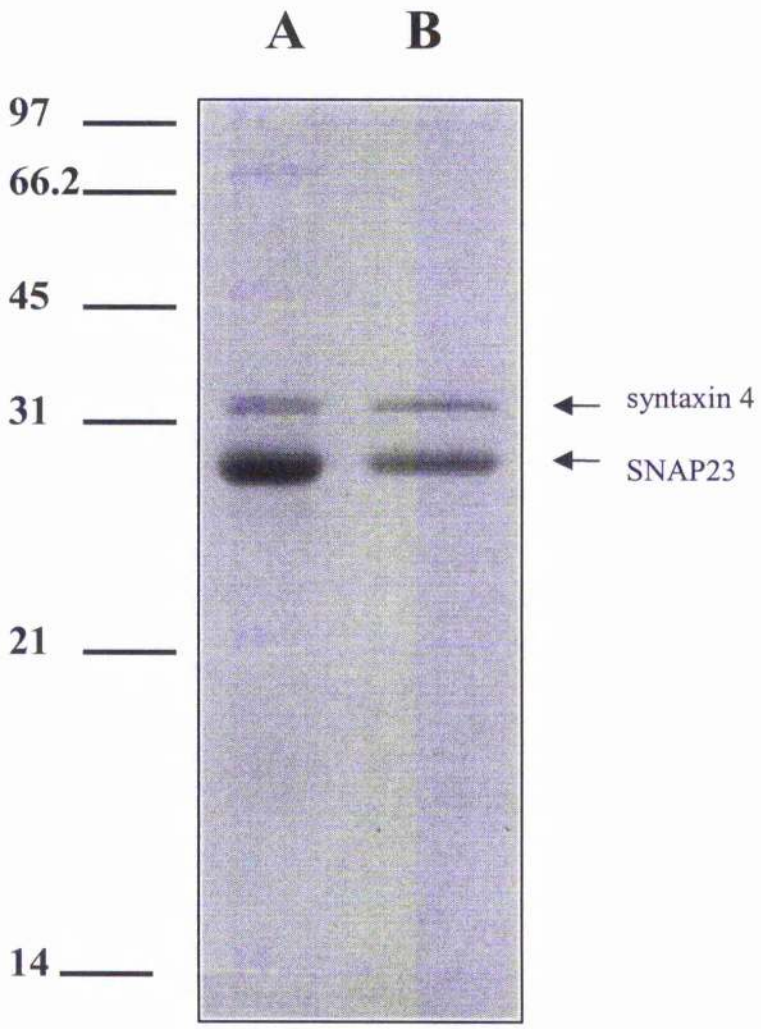


Figure 3.4: Reconstitution of t-SNAREs into liposomes.

Purified t-SNARE complex was successfully reconstituted into vesicles. The purified t-SNARE complex, consisting of syntaxin 4 and SNAP23 (A, 2 µl), along with t-SNARE complex that co-purified with vesicles (B, 10 µl) were separated on a 12 % SDS-PAGE gel and stained with Coomassie Blue as outlined in sections 2.2.1 and 2.2.4. Positions of the Bio-Rad low molecular weight markers are shown.

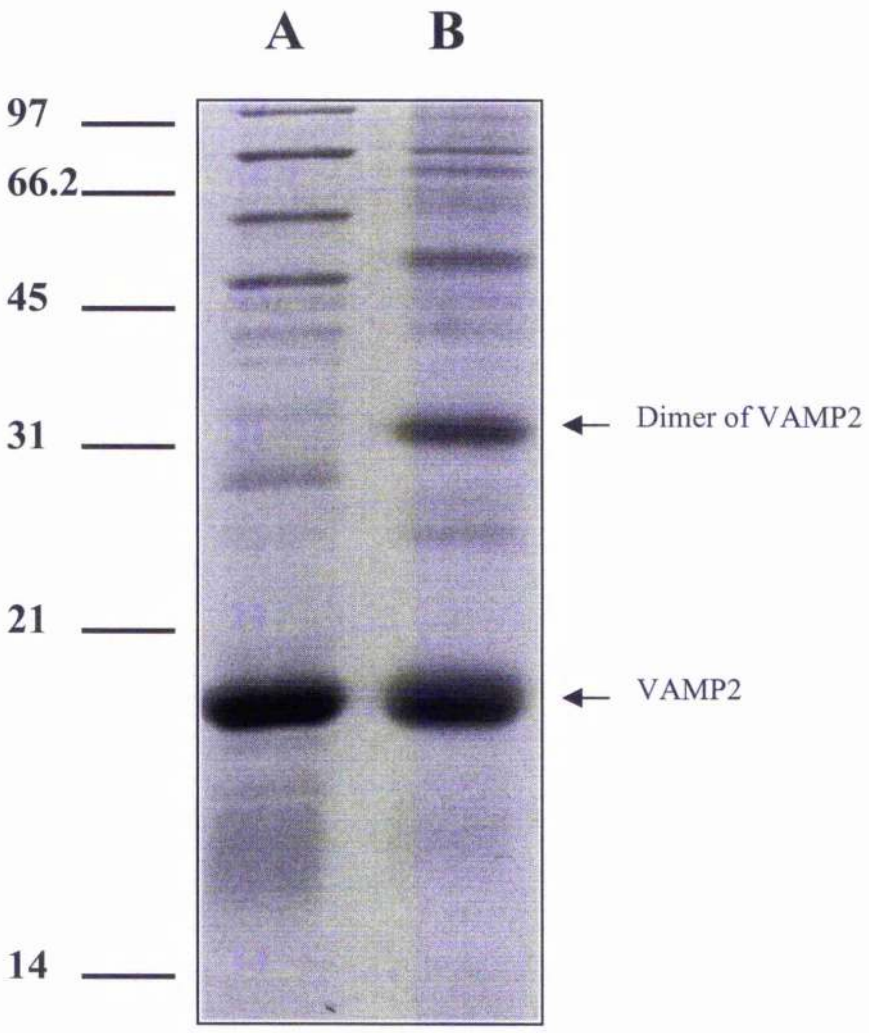


Figure 3.5: Reconstitution of v-SNAREs into liposomes

The purified v-SNARE protein (A, 2 µl) and the v-SNARE that co-purified with liposomes (B, 10 µl) were separated on a 15 % SDS-PAGE gel and stained with Coomassie Blue as outlined in sections 2.2.1 and 2.2.4. Positions of the Bio-Rad low molecular weight markers are shown.

Figure 3.4 shows the t-SNARE complex of syntaxin 4/SNAP23 was successfully reconstituted into liposomes. It can also be seen that flotation on the nycodenz gradient was successful in separating reconstituted t-SNARE complex from the excess soluble SNAP23, seen in the lower band in lane A.

Figure 3.5 shows the v-SNARE VAMP2 was successfully reconstituted into liposomes. As has previously been observed VAMP2 forms dimers, which run just above the 31 kDa marker, when reconstituted into liposomes (Weber *et al.*, 1998). This formation of dimers is thought to be mediated through the transmembrane region of VAMP2 (Laage *et al.*, 2000;Roy *et al.*, 2004).

Since both the t-SNARE complex and the v-SNARE had been successfully reconstituted into their appropriate liposome species it was possible to set up an *in vitro* fusion assay using these liposomes.

3.3.5 In vitro *fusion assay using reconstituted liposomes*

Reconstituted liposomes were used to set up an *in vitro* fusion assay as outlined in section 2.5.6. Briefly 45 µl of liposomes reconstituted with syntaxin 4/SNAP23, Figure 3.4 lane B, was added directly to the bottom of 2 duplicate wells of a microtitre plate on ice. Soluble VAMP2, 2 µl, Figure 3.2B, was added to the first well. To the other well 2 µl of glycerol free A200 was added to account for the volume difference. The plate was incubated on ice for 10 minutes prior to the addition of 5 µl of liposomes reconstituted with VAMP2, Figure 3.5 lane B, to each well. The top of the plate was sealed with tape and incubated at 4 °C overnight in the dark.

The following morning an additional two duplicate wells were set up in a similar manner just prior to commencing the fusion assay in order to see if pre-incubation at 4 °C affected the kinetics of fusion facilitated by these SNAREs.

NBD fluorescence was measured at 2 minute intervals for 2 hours in a fluoroskan II, with the excitation set at 460 nm and the emission measured at 535 nm, at a temperature of 37 °C. Following this two hour period, 10 µl of 2.5 % w/v n-dodecylmaltoside was added to

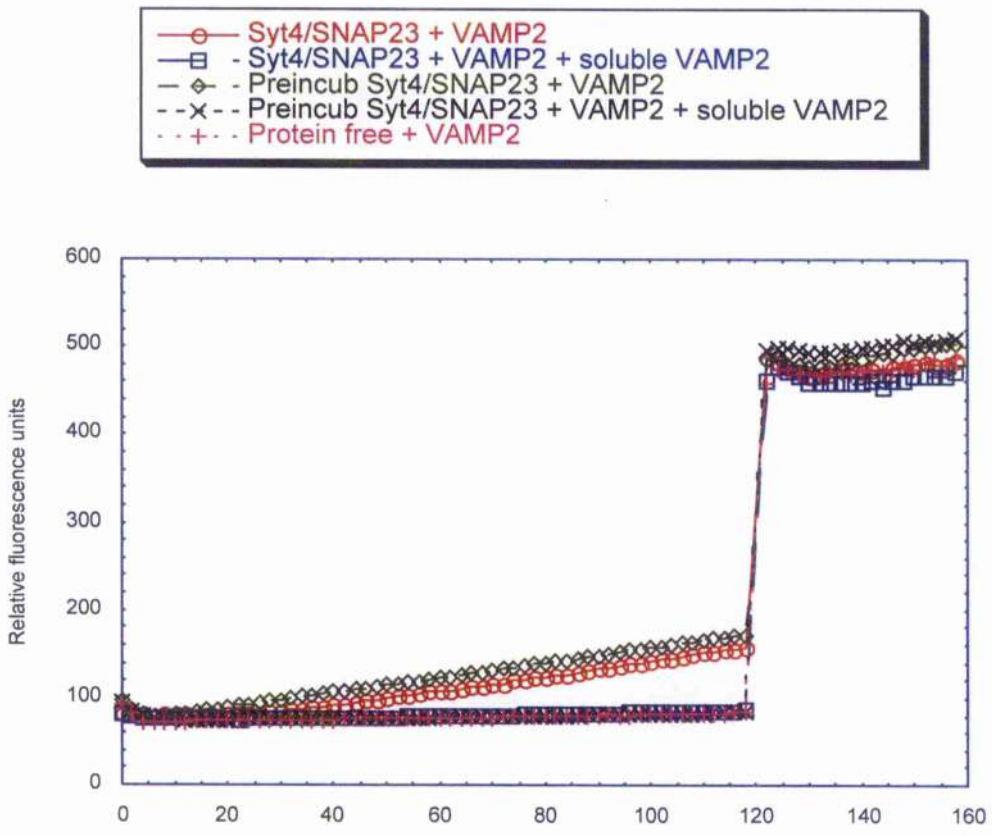
each well (to give NBD fluorescence at infinite dilution) and the plate mixed for 2 minutes. Fluorescence was then monitored for 40 minutes at 2 minute intervals.

Raw data from this experiment was plotted against time, as shown in Figure 3.6A, using KaleidaGraph. This was normalised to maximal detergent signal where this is represented by 100 %, as shown in Figure 3.6B. Using an equation from a calibration curve, performed by James McNew, of the NBD fluorescence given by different ratios of labelled lipids to unlabelled lipid this normalised data was converted into "rounds of fusion" (Parlati *et al.*, 1999;Scott *et al.*, 2003), as shown in Figure 3.6C. This calculation assumes that the two liposome populations which fuse are of approximately equal size. Liposomes are reconstituted with VAMP2 using lipid ratios of unlabelled to labelled that would result from zero rounds of fusion i.e. one labelled liposome, one round of fusion i.e. one labelled liposome fusing with one unlabelled liposome, two rounds of fusion i.e. one unlabelled liposome fusing with the previous resulting liposome, up to eight rounds of fusion. These liposomes are then monitored for NBD fluorescence at 535 nm before and after addition of n-dodecylmaltoside, normalised against the fluorescence given with just labelled liposomes (i.e. from the zero "rounds of fusion") before addition of detergent and % maximal NBD fluorescence calculated for each liposome population. A calibration curve is then plotted of normalised % maximal detergent signal for each liposome population with the y axis representing "rounds of fusion" and the x axis reprinting % maximal NBD fluorescence. A double exponential fitting of the plotted results gives the equation $Y = (0.49666 \times e^{(0.036031X)}) - (0.50597 \times e^{-0.053946X})$, where Y stands for "rounds of fusion" and X stands for % maximal detergent signal at any one time (Scott *et al.* 2003). Using this equation, % maximal detergent signal measured for each liposome population can be converted into rounds of fusion at each different time point. The data shown in Figure 3.6 is representative of 2 separate experiments.

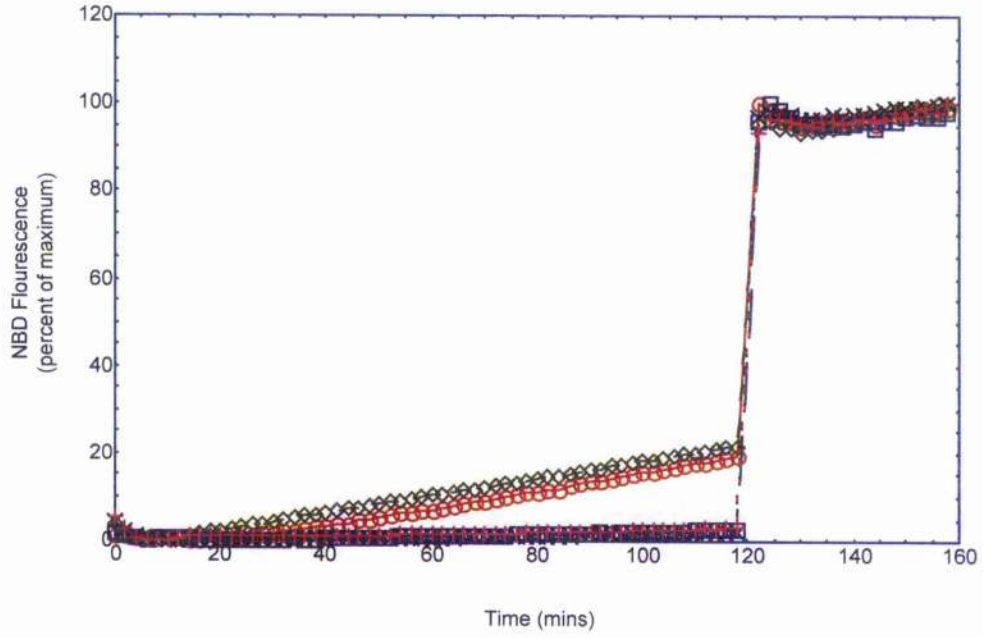
Figure 3.6: VAMP2 and syntaxin 4/SNAP23 form a functional complex capable of fusing liposomes

(A) Raw fluorescence data directly from the Fluoroskan II was plotted against time. Fusion assays were performed in which 5 µl of fluorescently labelled donor VAMP2 liposomes were mixed with 45 µl of unlabelled acceptor syntaxin 4/SNAP23 immediately before the assay, or pre-incubated overnight. **(B)** The raw Fluorescence was normalised to maximal detergent signal after addition of n-dodecylmaltoside (DM). **(C)** The percentage of maximal DM fluorescence was converted to "rounds of fusion", or fold lipid dilution by a calibration curve derived from the absorbance of dilutions of the two head group labeled lipids. As a control, the syntaxin 4/SNAP23 liposomes were preincubated for 10 minutes with soluble VAMP2 (+ soluble VAMP2).

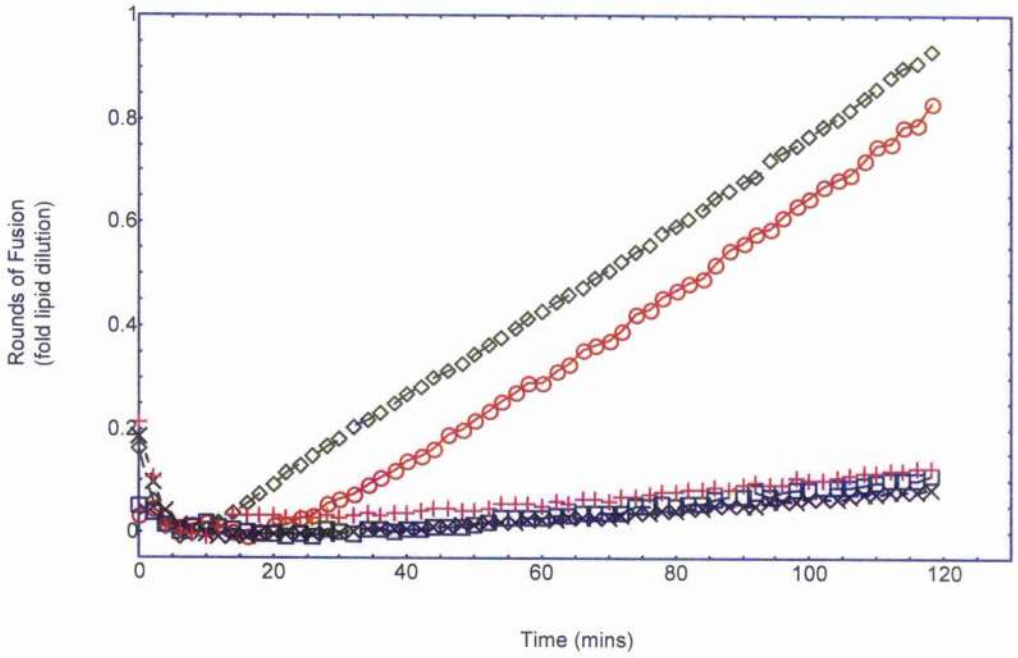
A



B


NBD Flourescence
(percent of maximum)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Time (mins)

C


Rounds of Fusion
(fold lipid dilution)
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
Time (mins)

Incubation of the fluorescent donor liposomes, reconstituted with VAMP2, with unlabelled liposomes, reconstituted with syntaxin 4/SNAP23, at 37 °C led to a time-dependant increase in NBD fluorescence (Red circles). Preincubation of these liposomes overnight at 4 °C (green squares) led to an increase in this fluorescence. As has been observed for the neuronal exocytic SNARE complex of syntaxin 1a/SNAP25 and VAMP2 (Weber *et al.*, 1998), but not for any of the yeast SNARE complexes, fusion is enhanced when liposomes are preincubated overnight at 4 °C. This increase in fusion has been proposed to be due to the formation of unfused intermediates where SNARE complexes are preformed (Weber *et al.*, 1998). The level of increase in fusion on preincubation of syntaxin 4/SNAP23 liposomes and VAMP2 liposomes was however, of a smaller magnitude than that observed for the neuronal exocytic SNARE complex (Parlati *et al.*, 1999).

As a control the soluble domain of VAMP2 was added to fusion reactions, both preincubated (black crosses) and unpreincubated (blue squares), in order to be sure that any fusion which was observed was protein mediated. This soluble VAMP2 forms complexes with the t-SNARE complex extremely quickly and thus blocks the formation of complexes between the two vesicle populations (Weber *et al.*, 1998). As can been seen in Figure 3.6C the addition of soluble VAMP2 did inhibit the fusion observed showing that any fusion is a result of the reconstituted SNARE proteins. As a further control to ensure that any fusion observed was mediated by the SNARE proteins, a fusion assay was carried out using VAMP2 acceptor liposomes and donor liposomes which were protein free. No fusion between these liposome populations was observed (pink crosses).

3.4 Discussion

The fusion process facilitated by SNARE proteins has been successfully reconstituted *in vitro* (Weber *et al.*, 1998). In this Chapter I have established an *in vitro* fusion assay for the SNAREs involved in the exocytosis of Glut4.

The t-SNAREs, syntaxin 4 and SNAP23, found on the plasma membrane of insulin responsive cells (Volchuk *et al.*, 1996;Tellam *et al.*, 1997;Wang *et al.*, 1997) were reconstituted into one population of liposomes, the acceptor. The v-SNARE VAMP2, which has been localised to Glut4-containing vesicles in insulin responsive cells (Volchuk *et al.*, 1997), was reconstituted into a separate population of liposomes, the donor. While the acceptor liposomes are composed of unlabelled lipid, the donor liposomes contain a small percentage of lipid labelled with either NBD or Rhodamine, at a concentration which leads to quenching of the NBD emission when excited with light at a wavelength of 485nm. When the liposomes fuse with each other these lipids are diluted in the membrane and fusion can thus be measured as increasing NBD emission. This assay is well established in the study of fusion facilitated by SNARE proteins (Scott *et al.*, 2003). Using this assay it was demonstrated that SNAREpins, that is the complex formed between cognate sets of v- and t-SNAREs, are necessary and sufficient to fuse artificial membranes (Weber *et al.*, 1998). This suggests that the formation of the energetically favourable SNARE complex is alone sufficient to overcome the repulsion between two membranes and facilitate fusion. Although VAMP2, syntaxin 4 and SNAP23 have all been implicated in Glut4 exocytosis in insulin-responsive cells and, have been shown by surface plasmon resonance to form a highly stable SDS-resistant SNARE complex *in vitro* (Rea *et al.*, 1998), they have until this study not been shown to form a SNAREpin capable of facilitating membrane fusion.

In order to set up the *in vitro* fusion assay using these SNAREs, they first needed to be purified from *E. coli*. The expression and purification of full-length VAMP2, which has been successfully purified by other groups (Weber *et al.*, 1998;Paraluti *et al.*, 1999) was relatively straight forward. The expression and purification of the syntaxin 4/SNAP23 complex for reconstitution proved to be more problematic, with syntaxin 4 being significantly insoluble under many of the conditions tested. By reducing the temperature at which expression was induced, a high enough yield of syntaxin 4 was obtained for reconstitution.

Fusion assays were carried out using the reconstituted donor and acceptor liposomes. Upon incubation at 37 °C, an increase in NBD fluorescence was observed, showing that these liposomes were capable of facilitating fusion. Preincubation of the liposomes overnight at 4 °C led to an increase in the level of fusion. This increase, upon preincubation, has previously been observed *in vitro* using the neuronal exocytic SNARE complex (Weber *et al.*, 1998;Parlati *et al.*,1999). However, in this case the increase was of a much larger magnitude (Weber *et al.*, 1998;Parlati *et al.*,1999). This increase is proposed to occur due to the predocking of the liposomes (Weber *et al.*, 1998;Parlati *et al.*,1999). A preincubation dependant increase in fusion has not been observed *in vitro* using yeast SNAREs (Scott *et al.*, 2003). Exocytic syntaxins have a large highly conserved N-terminal domain (Fernandez *et al.*, 1998) which, in some syntaxins has been shown to fold back on the SNARE domain holding it in a closed conformation (discussed in detail in Chapter 5). The removal of this N-terminal domain from the neuronal exocytic syntaxin 1a has been shown to accelerate fusion, without preincubation, *in vitro* (Parlati *et al.*, 1999) suggesting a role for the N-terminal in controlling docking of liposomes. The fact that preincubation of yeast SNAREs does not increase *in vitro* fusion (Scott *et al.*, 2003) suggests that in these SNAREs the N-terminal of syntaxin may not play such a role.

In order to confirm that any fusion of the liposomes was mediated by the reconstituted SNAREs, two controls were employed. The first control was the addition of soluble VAMP2, lacking the transmembrane domain, to acceptor liposomes prior to fusion (Weber *et al.*, 1998). This soluble VAMP2 binds the t-SNAREs, sequestering them from interaction with full-length VAMP2 on the donor membrane. The addition of soluble VAMP2 inhibited fusion facilitated by liposomes, which had been freshly mixed or preincubated overnight, showing that the fusion was mediated by the reconstituted SNAREs. A second control experiment was performed where the fusion of VAMP2 liposomes to protein free acceptor liposomes was monitored. No fusion was observed in this experiment, again showing that fusion was mediated by the SNARE proteins. Further controls, which could have been employed, include the addition of toxins which cleave the SNARE proteins. Tetanus toxin light chain, botulinum neurotoxin D and botulinum toxin B have all been shown to cleave free VAMP2 (Cheatham *et al.*, 1996;Macaulay *et al.*, 1997a;Macaulay *et al.*, 1997b;Foran *et al.*, 1999;Randhawa *et al.*, 2000). To show that the fusion mediated by the two liposome populations is facilitated by the reconstituted SNAREs, donor liposomes could be treated with one of these toxins prior to incubation with the acceptor liposomes.

In summary, in this Chapter I have successfully purified and reconstituted full-length syntaxin 4, SNAP23 and VAMP2 into liposome populations. The formation of syntaxin 4/SNAP23/VAMP2 ternary complex is sufficeint to directly fuse membranes, a fact thus far assumed but not definitively shown. This assay can now be used to study the effects of different factors on the fusion mediated by these SNARE proteins.

Chapter 4

Affect of DRM lipids on fusion and the generation of recombinant baculovirus to purify palmitoylated SNAP23 in complex with syntaxin 4

4.1 Introduction

Recently SNARE proteins have been localised to lipid microdomains of the plasma membrane termed lipid rafts in a number of different cells. A significant proportion of the t-SNAREs, syntaxin 4 and SNAP23, and the v-SNARE, VAMP2, involved in Glut4 exocytosis in insulin-responsive cells have been isolated in lipid rafts purified as detergent resistant membranes (DRMs) from 3T3-L1 adipocytes (Chamberlain and Gould, 2002). Other proteins known to be regulators of SNARE function including Munc 18c were found to be excluded from these domains (Chamberlain and Gould, 2002). Whether targeting of these SNARE proteins to rafts regulates the fusion facilitated by these SNAREs is yet to be established.

It has been shown that in PC12 cells the targeting of the SNAP23 homologue SNAP25 to detergent resistant membranes, which appears to be dependent on the palmitoylation status of this molecule, effects the ability of the neuronal exocytic SNARE complex to support fusion (Salaun *et al.*, 2005a). Palmitoylation, which is reversible, is therefore a potential way to regulate SNARE complex formation and fusion.

4.1.1 Regulation of fusion by partitioning into raft domains

Lipid rafts have been shown to be involved in a viral entry into a number of cells (reviewed in Rawat *et al.*, 2003). The mechanism of fusion facilitated by viral fusion proteins has been speculated to share many similarities with that of the fusion facilitated by SNARE proteins (Sollner, 2004). While certain proteins are found to be enriched in lipid raft domains others seem to be excluded. This selective recruitment of proteins makes these domains ideal to spatially regulate fusion. Components of the insulin signalling pathways (along with the SNAREs involved in Glut4 exocytosis) have also been localised to these domains, suggesting that in insulin-responsive cells these domains might spatially regulate fusion.

Many studies have used the agent methyl-β-cyclodextrin that extracts cholesterol from the membrane to study the function of rafts. Two independent studies showed that exocytosis was inhibited in cells treated with this agent (Chamberlain *et al.*, 2001;Lang *et al.*, 2001), suggesting that cholesterol enriched domains may be the site of exocytosis within the cell.

However, these studies must be treated with caution as cholesterol depletion may have other effects within the cell (Munro 2003;Rodal *et al.*, 1999).

4.1.2 Targeting of SNAREs to raft domains

The addition of palmitate groups to proteins has been suggested to influence their targeting to raft domains (Brown, 2006). Palmitate groups are saturated, and have therefore been proposed to partition into raft domains due to favourable packing (Brown and London, 1998). There are several studies that provide evidence for the importance of palmitoylation of proteins in targeting to lipid rafts. Blocking the palmitoylation of the protein Fyn using 2-Bromopalmitate significantly reduced its association with DRMs in COS and Jurkat cells (Webb *et al.*, 2000), while blocking the palmitoylation of α0 subunit of the heterotrimeric Gi-like protein by site directed mutagenesis of a cysteine residue similarly reduced the affinity of this protein for the detergent insoluble fraction in COS 7 cells (Guzzi *et al.*, 2001). In Madin Canine Kidney cells, a high proportion of raft proteins, isolated along with DRMs, were found to be labelled with ^{3}H palmitate (Melkonian *et al.*, 1999). However, as not all palmitoylated proteins were found to be targeted to lipid rafts palmitoylation does not seem to be a universal signal for association with lipid raft domains (Melkonian *et al.*, 1999).

SNAP25 and SNAP23 are both palmitoylated on a conserved cysteine rich domain in the linker region between their two SNARE domains (Lane and Liu, 1997;Vogel and Roche, 1999). In SNAP25, four cysteines in this domain are thought to be palmitoylated, while in SNAP23 palmitoylation seems to occur on five cysteines in the same conserved domain. The palmitoylation state of SNAP25 and SNAP23 influence their targeting to raft domains in PC12 cells, with the five cysteine motif of SNAP23 supporting a higher level of association of the protein with these domains than the four cysteine motif of SNAP25 (Salaun *et al.*, 2005b). A subsequent study showed a negative correlation between the extent of SNAP25/23 associated with DRMs and the level of exocytosis that was supported (Salaun *et al.*, 2005a). Whether cycles of palmitoylation and depalmitoylation represent a regulatory mechanism of SNAP25/23 raft localisation and therefore regulate the ability of the SNARE complex to support fusion remains to be established.

While raft association of SNAP25 and SNAP23 appears to be controlled by palmitoylation, the mechanism controlling the association of syntaxin with raft domains is less clear. It

has been suggested that perhaps the transmembrane domain of syntaxin may preferentially partition into raft domains due to its ability to interact with cholesterol (Lang *et al.*, 2001). However, recombinant syntaxin does not localise into cholesterol/sphingolipid rich domains in reconstituted giant unilamellar vesicles (Bacia *et al.*, 2004), or supported bilayers (Saslowsky *et al.*, 2002). More recently it has been suggested that syntaxin 1a may associate with the lipid species $PI4,5P_2$, which has been shown to be enriched in raft domains (Aoyagi *et al.*, 2005).

The v-SNAREs VAMP2, Snc1p and Snc2p have been shown to be palmitoylated on a conserved cysteine residue adjacent to the transmembrane domain (Couve *et al.*, 1995;Veit *et al.*, 2000). Recently a proteomic approach to identify palmitoylated proteins in yeast found 47 proteins modified with palmitate, 12 of which where already known to be palmitoylated (Roth *et al.*, 2006). This screen pulled out 8 SNARE proteins, including Snc1p and Snc2p, all of which have juxtamembrane cysteine residues (Roth *et al.*, 2006). The physiological significance of the addition of palmitate to these SNAREs is yet to be characterised.

4.1.3 Palmitoylation

Mammalian proteins are commonly post translationally modified by the addition of lipid moieties (reviewed in Resh, 1996). S-palmitoylation is the addition of a palmitate group (C16:0) to a cysteine residue within a protein through a reversible covalent thioester bond (Bijlmakers and Marsh, 2003). A thioester bond is formed when a sulfhydryl –SH group reacts with a carboxylic acid group –COOH, with the release of a molecule of water, to form a thioester –S-CO- group. This particular post-translational modification is wide-spread and is found almost exclusively on membrane associated proteins. In some cases it attaches otherwise soluble proteins to the membrane while in others it is attached to proteins which contain transmembrane domains (Bijlmakers and Marsh, 2003). Although palmitoylation can occur on internal sequences the addition of palmitate to otherwise soluble proteins typically occurs at either the C or N terminus. Proteins that are inserted into the membrane are generally palmitoylated at the juxtamembrane region (Bijlmakers and Marsh, 2003).

The addition of palmitate groups to proteins is facilitated by a group of proteins called palmitoyl acyltransferases within the cell (reviewed in Dietrich and Ungermann, 2004).

Apart from the requirement for a cysteine residue, a consensus sequence for the addition of palmitate to a protein has not been characterised. The compartmental site of palmitate addition to proteins within the cell by palmitoyl acyltransferases is still a matter of debate. While the addition of some lipid moieties e.g. myristoylation or prenylation is usually irreversible, the addition of palmitate groups is reversible, and subsequently could be used as a dynamic means to control protein function (Bijlmakers and Marsh, 2003). Depalmitoylation is believed to occur through the action of palmitoyl thioesterases, but only a limited number of these enzymes have so far been identified in mammalian cells (reviewed in Huang and El Husseini, 2005). So far the enzymes that add and remove palmitate groups from SNAP23 have remained elusive.

4.1.4 Palmitoylation and fusion

Palmitoyl acyltransferase activity has been observed at various locations within the cell (Mitchell *et al.*, 2006) . The cellular site for the palmitoylation of SNAP25 is unknown. Palmitoylation does require a functional secretory pathway, as the addition of palmitate to SNAP25 is prevented by treatment with Brefeldin A (Gonzalo and Linder, 1998). This suggests that SNAP25 is palmitoylated post ER either in the Golgi, or at the plasma membrane, or that the palmitoylation of SNAP25 requires another protein which traffics through the Golgi.

While some studies have suggested that the palmitoylation of SNAP25 homologues provides membrane targeting of this otherwise hydrophilic protein, chemical deacylation of the protein does not lead to displacement from the membrane, suggesting this may not be the case (Gonzalo and Linder, 1998). This suggests that the membrane association of SNAP25 may be due to association with other membrane constituents, the most likely candidate being syntaxin or membrane lipid. This is supported by the fact that the yeast homologue of SNAP25, Sec9, and SNAP29 are found associated with the membrane despite the fact that they have no palmitate groups or transmembrane domain (Brennwald *et al.*, 1994;Steegmaier *et al.*, 1998). This has been supported by pulse-chase experiments which showed that SNAP25 first associates with syntaxin before the t-SNARE complex enters the membrane (Vogel *et al.*, 2000). In addition, removal of the central cysteine domain did not stop this interaction, or the membrane association of SNAP25, suggesting that SNAP25 becomes palmitoylated following recruitment to the membrane by syntaxin (Vogel *et al.*, 2000).

Palmitoylation of SNAP25 does not seem to influence the ability of this SNARE to enter into SNARE complexes (Washbourne *et al.*, 2001). However, mutation of the cysteine residues in SNAP25, and therefore blockage of palmitoylation, prevented rescue of exocytosis in PC12 cells treated with BoNT/E, compared to unmutated SNAP25, suggesting an important role for palmitoylation in fusion (Washbourne *et al.*, 2001). However, in HIT cells, similar mutants were able to rescue some degree of exocytosis (Gonelle-Gispert *et al.*, 2000). Furthermore, in a cracked PC12 cell assay, the introduction of recombinant SNAP25 produced in *E. coli,* which is unpalmitoylated, was able to rescue calcium dependent exocytosis: However, this study did not compare this to the level of rescue achievable with the palmitoylated protein (Scales *et al.*, 2000). A role for palmitoylation in the regulation of fusion has also been suggested by the observation that vacuole to vacuole fusion is stimulated by palmitoyl-CoA in an *in vitro* fusion assay (Haas and Wickner, 1996). The addition of palmitoyl-CoA also stimulates the fusion of vesicles to the Golgi *in vivo* (Pfanner *et al.*, 1990).

4.1.5 Post-translational modification of recombinant proteins

For the majority of studies involving SNAREs, recombinant protein has been expressed in, and purified from, *E. coli*. While the use of *E. coli* has a number of advantages, such as cost and ease of scale-up, *E. coli* cells, as prokaryotes, are incapable of many of the post translational modifications which normally occur in mammalian cells. As mentioned above, SNAP23 is covalently modified in mammalian cells by the addition of palmitate groups. Baculovirally driven expression of protein within insect cells, a eukaryotic host, offers a way to obtain near-authentically processed recombinant proteins.

4.1.6 Baculoviruses

Baculoviruses, insect viruses that predominantly infect butterflies and moths, have been used to produce a great number of recombinant proteins. Over 500 types of baculovirus have been identified however, the baculovirus most commonly used for foreign gene expression is the baculovirus *Autographa californica* multiple NPV (AcMNPV) (HU, 2005). Baculovirally infected insect cells have many advantages over other systems used for the production of recombinant protein. In the majority of cases, since insect cells are

higher eukaryotes, proper post-translational modifications of the recombinant proteins occur (HU, 2005). Baculoviruses have large 130 kb dsDNA genomes, allowing large inserts to be engineered into it (HU, 2005). Baculoviruses also have a very restricted host range and do not replicate in mammalian cells, making them non-hazardous and easy to propagate. The promoters commonly used to drive the production of recombinant proteins are very strong which can give high yields of recombinant protein with protein expression levels often similar to, or higher than, those seen in prokaryotic systems.

Baculoviruses have a biphasic life cycle with two viron forms, polyhedra and budded virus (reviewed in Okano *et al.*, 2006). Viruses of the genus nucleopolyhedrovirus (NPV) produce large proteinaceous polyhedron-shaped occlusion bodies (2 μm -15 μm in size), called polyhedra that contain multiple virions (membrane-enveloped nucleocapsids). During the natural infectivity cycle AcMNPV, occluded in polyhedra, is ingested by the insect, the poyhedra broken down by the alkaline environment of the midgut, and the virions are released. These virions are then free to infect the epithelial cells of the gut. In these cells the DNA genome is replicated and transcribed in the nucleus and new virions are synthesised. Baculovirus gene expression is regulated at the transcriptional level and can be separated into three phases: early, late and very late. These virions then bud from the initially infected cell through the plasma membrane, giving rise to budded virus and subsequently lead to a systemic infection of the insect host. Budded virus is highly infectious to cultured insect cells and is the main form used for protein production.

The late genes encode for proteins involved in the lysis of the host cell and the survival of the virus. During the natural viral life cycle large amounts of these proteins are produced. In tissue culture these genes are non-essential and their promoters can be used to drive the high expression of recombinant proteins. The two main promoters used for the production of recombinant protein are p10 and PH (Beljelarskaya, 2002). The PH promoter drives the expression of the protein poyhedrin, the major component of polyhedra. It is the second most abundant protein in the infected cell late in infection. The p10 promoter is also non-essential in insect cell culture, although its gene product is less well understood. Deletion of these genes reduces background problems caused by the expression of large amounts of endogenous protein while increasing the expression of foreign proteins.

The initial baculoviral systems used to produce recombinant protein relied on an inefficient homologous recombination event. Since the viral genome is too large to allow easy

foreign gene insertion through conventional cloning, a transfer vector must be used. Insect cells, from *Spodoptera frugiperda* (Sf), were transfected with both the transfer vector and the bacmid encoding the baculovirus genome. The rates of recombination were estimated at 0.1 %, and recombined bacmid had to be separated from parental bacmid by a series of technically difficult and time consuming plaque assays. Although the method was improved by using linearised viral DNA to give recombination rates of upto 90 % the isolation of recombinant baculovirus still required plaque assays (Kitts and Possee, 1993). This step has since been cut out with a new system to produce recombinant baculovirus. In this system the transposition step is carried out in *E. coli* cells that stably express both the bacmid DNA and a helper plasmid providing proteins to enable the transposition upon introduction of the transfer vector. From these *E. coli* cells recombinant bacmid can be isolated and this is then used to transfect insect cells to ultimately produce the baculovirus (Luckow *et al.*, 1993). This method of transposition was first described in 1993 by Luckow and colleagues (Luckow *et al.*, 1993), and has since been commercialised by Invitrogen as the Bac-to-Bac system.

4.1.7 Baculovirus protein production and palmitoylation

Many recombinant proteins produced using the baculoviral system appear to be correctly folded and post-translationally modified. Most post-translational modifications appear to be carried out in the same way in insect cells as in mammalian cells, with the exception of glycosylation. These modifications include the addition of fatty acyl chains including palmitate. The addition of palmitate groups to recombinant proteins has been shown to occur in insect cells (Pickering *et al.*, 1995;Ponimaskin *et al.*, 2001). Recently it has been shown that SNAP25 expressed using baculoviral infection of Sf9 cells is palmitoylated (Kammer *et al.*, 2003).

4.2 Aims of this chapter

The t-SNAREs syntaxin 4 and SNAP23, along with the v-SNARE VAMP2, involved in the exocytosis of Glut4 containing vesicles, have been found in detergent resistant membranes isolated from 3T3-L1 adipocytes (Chamberlain and Gould, 2002). In order to assess whether the main lipids, cholesterol and sphingomyelin, found in lipid rafts have any effect on fusion, these lipids were incorporated into liposomes which were used in the fusion assay outlined in Chapter 3.

Although non-palmitoylated SNAREs purified from *E. coli* are capable of facilitating fusion *in vitro* when reconstituted into liposomes (Weber *et al.*, 1998), the rate of fusion observed is substantially slower than that observed *in vivo* (Wolfel and Schneggenburger, 2003). It is possible that the acyl chains on SNAP25 and SNAP23 may perturb the membrane *in vivo*, and thus facilitate fusion. Recently it has been shown that SNAP25 expressed in, and purified from, insect cells is acylated (Kammer *et al.*, 2003). A baculovirus was engineered to allow expression and purification of syntaxin 4 and SNAP23 from insect cells.

4.3 Methods and Results

4.3.1 Reconstitution of SNAREs into cholesterol and sphingomyelin containing liposomes and fusion using these liposomes

Lipid rafts are specialised regions of the lipid bilayer that are known to be enriched in certain lipids including sphingomyelin and cholesterol. In order to investigate the effect of these lipids on fusion facilitated by SNARE proteins they were incorporated into t-SNARE containing liposomes that were subsequently used for the fusion assay as outlined in Chapter 3.

Liposomes were reconstituted with t-SNAREs as outlined in sections 2.5.3-2.5.4. In order to investigate whether the inclusion of detergent resistant membrane lipids influenced fusion supported by these SNAREs, liposomes were produced with the standard lipid composition (85 mol% POPC/15 mol% DOPS), 33 mol% cholesterol/52 mol% POPC/15 mol% DOPS and 33 mol% cholesterol/20 mol% sphingomyelin/32 mol% POPC/15 mol% DOPS. The lipid recovery of each t-SNARE population was similar as judged by the trace amounts of [^{3}H]-DPPC added to the lipid mix. Fusion assays were carried out as outlined in section 2.5.6, and data analysed as outlined in section 2.5.7. The data shown in Figure 4.1 is representative of 2 separate experiments.

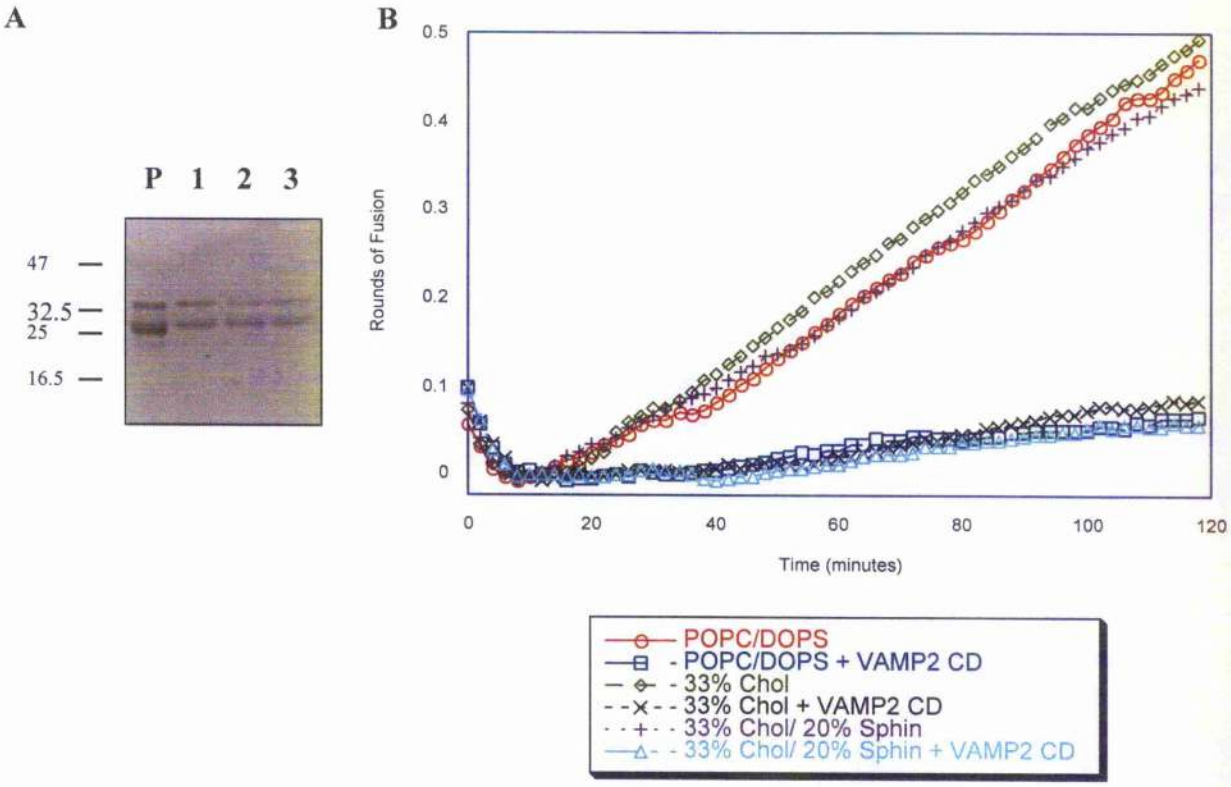


Figure 4.1: Affect of cholesterol and sphingomyelin on fusion facilitated by syntaxin 4, SNAP23 and VAMP2

(A) Syntaxin 4 and SNAP23 purified as outlined in section 2.5.1 (P) were reconstituted into liposomes containing (1) 85 mol% POPC/15 mol% DOPS, (2) 33 mol% cholesterol/52 mol% POPC/15 mol% DOPS (3) 33 mol% cholesterol/20 mol% sphingomyelin/ 32 mol% POPC/15 mol% DOPS as outlined in sections 2.5.3 and 2.5.4. Purified protein (2 µl) and liposomes (10 µl) were run on a 12 % SDS-PAGE gel as outlined in section 2.2.1 and stained with Coomassie blue as outlined in section 2.2.4. Positions of broad range molecular markers shown. **(B)** Reconstituted liposomes were used to perform a fusion assay as outlined in section 2.5.6. The data was converted to rounds of fusion as outlined in section 2.5.7. POPC/DOPS = liposomes of standard lipid compostition, 33% Chol = liposomes of 33 mol% cholesterol/52 mol% POPC/15 mol% DOPS, 33% Chol/20% Sping = liposomes of 33 mol% cholesterol/20 mol% sphingomyelin/32 mol% POPC/15 mol% DOPS. VAMP2 CD indicates that t-SNARE containing liposomes were first preincubated with the soluble domain of VAMP2.

From Figure 4.1A, it can be seen that the t-SNAREs syntaxin 4 and SNAP23 were reconstituted into the different liposome populations to approximately equal levels. The fusion assay, shown in Figure 4.1B, shows that the inclusion of either 33 mol% cholesterol or 33 mol% cholesterol and 20 mol% sphingomyelin appears to have no effect on the rate of fusion of these liposomes with liposomes containing VAMP2.

These data were unexpected due to the difference in lipid compositions of the liposomes involved. The calculation from raw data to rounds of fusion, as explained in Chapter 3, assumes that the two liposome populations which are fusing are of approximately equal size, it was therefore decided to investigate whether the inclusion of these lipids had any effect on the size of the t-SNARE liposomes produced.

4.3.2 *Sizing of cholesterol and sphingomyelin containing liposomes*

The size of the t-SNARE liposomes was analysed by Transmission Electron Microscopy as described in section 2.5.5. The results are presented in Figure 4.2.

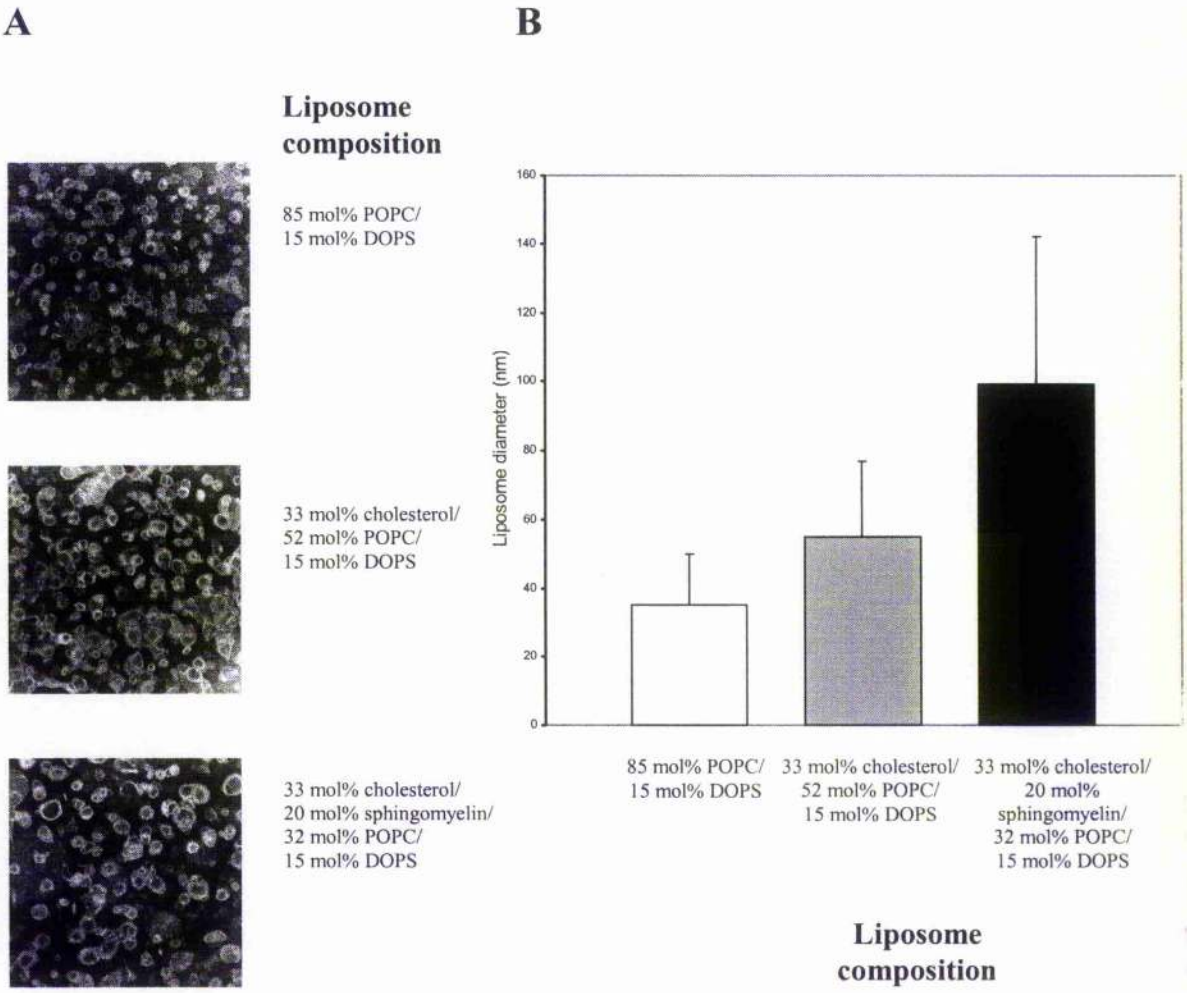


Figure 4.2: Sizes of reconstituted t-SNARE liposomes

t-SNARE liposomes were reconstituted containing 85 mol% POPC/15 mol% DOPS, 33 mol% cholesterol/52 mol% POPC/15 mol% DOPS and 33 mol% cholesterol/20 mol% sphingomyelin/32 mol% POPC/15 mol% DOPS as outlined in sections 2.5.3 and 2.5.4. These were sized by TEM as outlined in section 2.5.5. (A) Representative EM images. (B) The sizes of the liposomes +/- standard deviation (n=310 for each liposome species, $p<0.05$)

The inclusion of these lipid species obviously has a dramatic effect on the size of the liposomes produced. Since the inclusion of cholesterol and cholesterol/sphingomyelin significantly increased the size of the t-SNARE liposomes produced, the rounds of fusion calculated for these liposomes will be overestimated due to the much greater surface area produced and therefore much higher NBD fluorescence on fusion with one v-SNARE liposome. The inclusion of cholesterol and sphingomyelin into the t-SNARE liposomes therefore appears to be inhibitory to fusion however, in order to directly compare this data new calibration curves using the resulting ratios of lipids from one round of fusion etc, as outlined in section 3.2.5, would have to be generated. This was unfortunately out with the time constraints of this project.

4.3.3 Generation of recombinant baculovirus to express full-length syntaxin 4 and SNAP23 in Sf9 cells

Although it has been demonstrated, *in vitro,* that SNAP25 purified from *E. coli* is palmitoylated in the absence of an enzyme upon addition of [^{3}H]pal-CoA and that this palmitoylation is increased on association of SNAP25 with syntaxin 1A, the estimated levels of SNAP25 palmitoylated were less than 3 % (Veit, 2000). Recently it has been shown the majority of SNAP25 purified from insect cells infected with a baculovirus expressing SNAP25 was palmitoylated on the central cysteine region (Kammer *et al.*, 2003). It was therefore decided to generate a recombinant baculovirus to co-express full-length syntaxin 4 and SNAP23 in Sf9 cells. In order to generate this baculovirus the Bac-to-Bac system supplied by Invitrogen was used.

There are 3 main stages involved in creating a recombinant baculovirus, as outlined in Figure 4.3. These are:

4.3.3.A Cloning of gene of interest into pFastBac^{TM} Dual

4.3.3.B Homologous recombination of pFastBac^{TM} Dual and the baculovirus backbone vector in *E. coli*

4.3.3.C Production of baculovirus particles in Sf9 cells

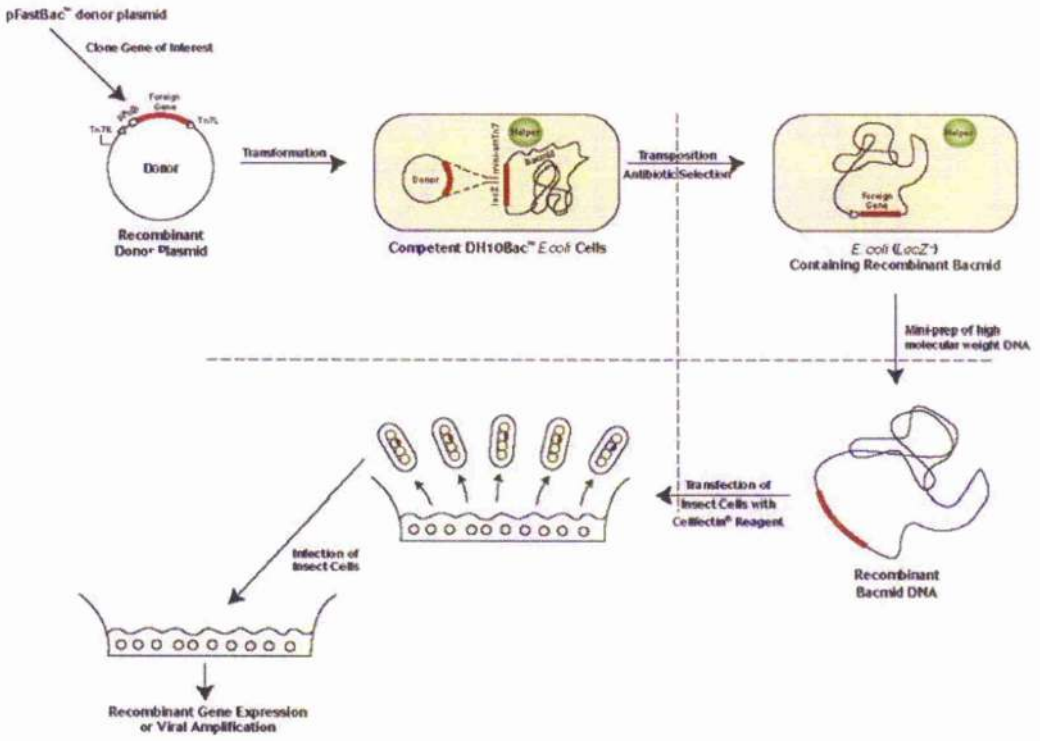


Figure 4.3: The steps followed to generate recombinant baculovirus

(adapted from the Bac-to-Bac manual)

Step 1: Cloning of gene of interest into pFastBac^{TM} Dual

Step 2: Homologous recombination of pFastBac^{TM} Dual and the baculovirus backbone vector in *E. coli*

Step 3: Production of baculovirus particles in Sf9 cells

The methods used are described in sections 4.4.3.A, 4.4.3.B and 4.4.3.C.

4.3.3.A *Cloning of genes into pFastBac™ Dual*

Baculoviral transfer vectors typically allow for the expression of single recombinant proteins in one insect cell. Multiprotein complexes have been produced by coinfection of insect cells with multiple baculoviruses, but the reproducibility of this is difficult to achieve. The development of a transfer vector capable of supporting the expression of two proteins simultaneously has allowed the coexpression of the proteins within one insect cell. pFastBac™ Dual contains two multiple cloning sites, each under the control of two separate promoters: p10 and PH. The left and right arms of Tn7, which enables recombination with the baculovirus shuttle vector, flank the expression cassette containing these two multiple cloning sites and promoters. The vector pFastBac™ dual is a non-fusion vector, therefore when cloning the gene of interest into this vector a start ATG site and a stop codon for termination must be engineered into the insert.

GST-SNAP23

Since the vector pET41a, which SNAP23 had previously been cloned into, had complementary restriction sites prior to the GST tag and after the termination of the SNAP23 sequence GST-SNAP23 could be conveniently excised in whole and cloned directly into pFastBac™ Dual. GST-SNAP23 was ligated into the multiple cloning site under the control of the polyhedron promoter using the restriction sites *Hind* III and *Xba* I. GST-SNAP23 was first excised from pET41a by restriction digest with *Hind* III and *Xba* I as outlined in section 2.3.5. The fragment was run on an agarose gel as outlined in section 2.3.3, shown in Figure 4.4A, and gel purified as outlined in section 2.3.4. The purified fragment was subsequently ligated, as outlined in section 2.3.7, into the pFastBac™ Dual vector which had been cut with the appropriate restriction enzymes, as shown in Figure 4.4A, and gel purified as outlined in section 2.3.4. Following ligation the DNA was transformed into DH5α cells, as outlined in section 2.3.9. Colonies were minipreped, as outlined in section 2.3.10, and screened for the presence of insert by restriction digest with *Xba I* and *Hind III*, as shown in Figure 4.4B.

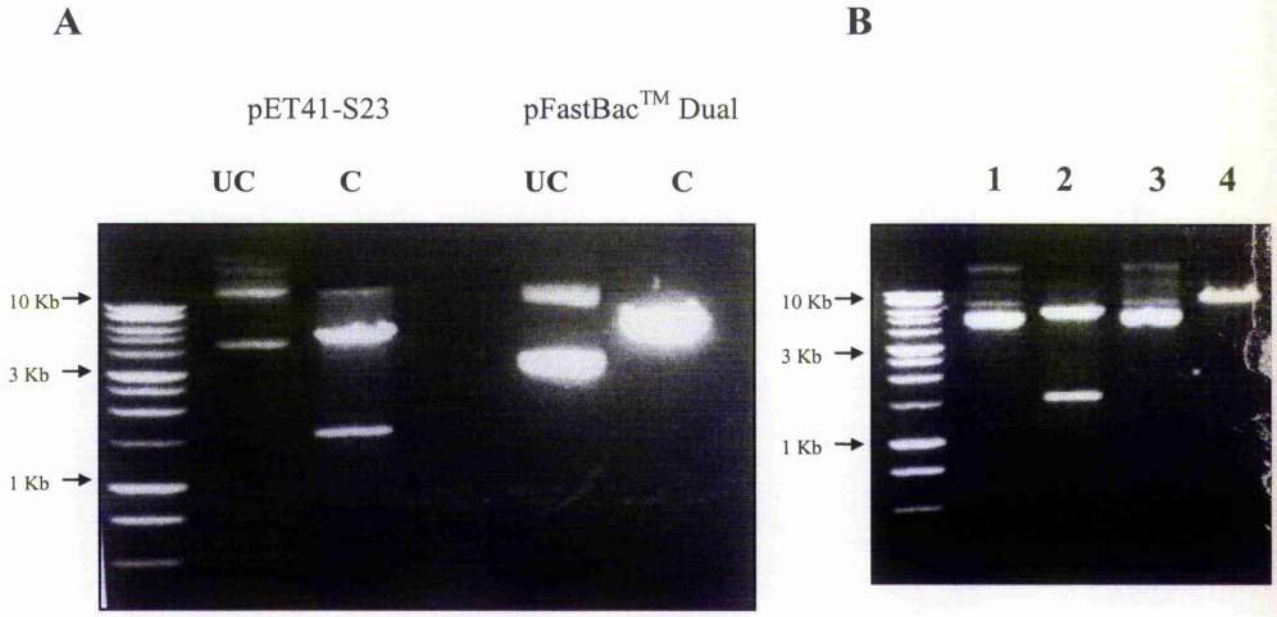


Figure 4.4: Cloning of GST-SNAP23 into pFastBac™ Dual and digestion for subsequent cloning

(A) Restriction digest of pET41a-SNAP23 and pFastBac™ Dual with *Hind* III and *Xba* I prior to ligation of pFastBac™ Dual and GST-SNAP23. The Band between the 3 kb and 10 kb marker is cut pET41a, the band running at roughly 1.5 kb is GST-SNAP23. Cut pFastBac™ Dual gave a band of the expected size (B) Restriction of pFastBac™ Dual with *Hind* III and *Xba* I to identify presence of insert and digestion using *Nhe* I and *Xho* I for subsequent ligation with syntaxin 4. lane 1 – uncut pFastBac™ Dual-S23, lane 2 - pFastBac™ Dual-S23 digested with *Hind* III and *Xba* I. The band running at roughly 1.5 kb is GST-SNAP23, lane 3 – uncut pFastBac™ Dual-S23, lane 4 - pFastBac™ Dual-S23 digested *Nhe* I and *Xho* I.

UC indicates lanes containing uncut plasmid while C indicates lanes containing cut plasmid. The positions of the Promega 1 kb DNA ladder are indicated.

Full-length syntaxin 4

Full-length syntaxin 4 was amplified by PCR from pQE30 using the forward primer 5'**CTCGAG**ATGCGCGACAGGACCCACG 3' and the reverse primer 5'**GCTAGC**TTATCCAACGGTTATGGTG 3' as outlined in section 2.3.1 (restriction sites *Xho* I and *Nhe* I were incorporated into the primers and are shown in bold respectively). Following PCR the fragment, which was of the appropriate size was excised from an agarose gel, gel purified and TA cloned into pCRII-TOPO as outlined in section 2.3.6. Colonies were screened for the presence of insert by digestion with *Nhe* I and *Xho* I. Colonies which contained insert were sequenced to ensure error free sequence as outlined in section 2.3.12.

The restriction enzymes *Nhe* I and *Xho* I were used to digest the pFastBac™ Dual vector containing GST-SNAP23 (shown in lane 4 of Figure 4.4B) and pCRII-TOPO containing full-length syntaxin 4 (shown in Figure 4.5A) as outlined in section 2.3.5. Appropriate fragments were gel purified, ligated and transformed into DH5α as outlined in sections 2.3.4, 2.3.7 and 2.3.9. Colonies were minipreped as outlined in section 2.3.10 and screened for the insertion of DNA encoding full-length syntaxin 4 by restriction digest using the restriction enzymes *Nhe* I and *Xho* I as outlined in section 2.3.5, shown in Figure 4.5B.

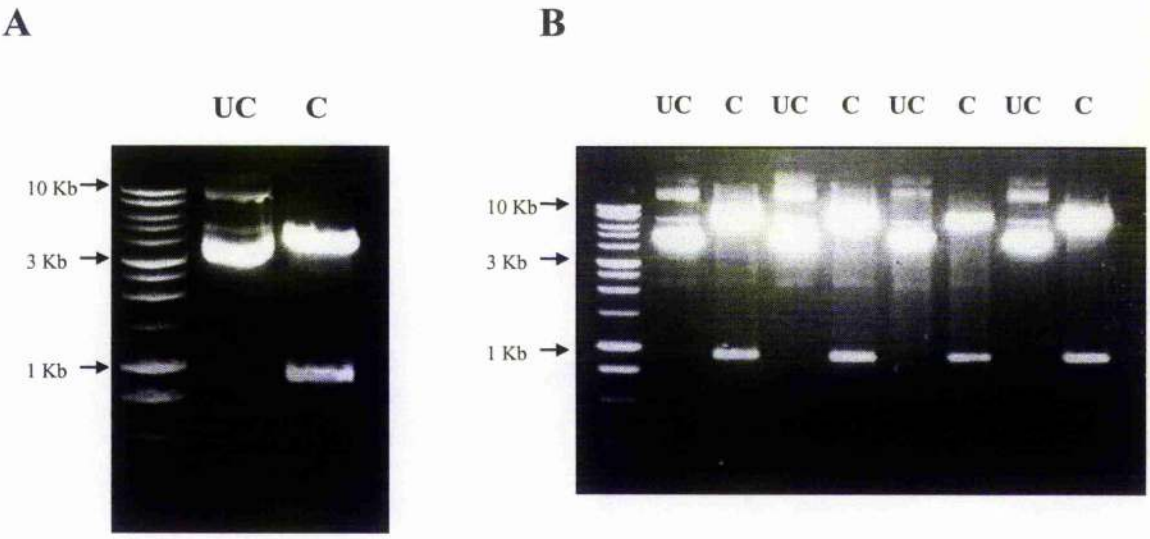


Figure 4.5A: Restriction of pCRII-TOPO ligated with syntaxin 4 to confirm presence of and isolate insert for subsequent cloning into pFastBac™ Dual containing GST-SNAP23 (pFastBac™ Dual-S23)

Restriction digest of pCRII-syntaxin 4 with *Xho* I *and Nhe* I prior to ligation. The Band running between the 3 and 4 kb marker is cut pCRII-TOPO while the band running just below the 1 kb marker is syntaxin 4.

Figure 4.5B: Restriction of pFastBac™ Dual-S23 /S4 with Xho I and Nhe I to confirm the presence of syntaxin 4

Colonies from the ligation of gel purified syntaxin 4 and pFastBac ™ Dual-S23 fragments were minipreped and screened for the presence of syntaxin 4 by restriction digest with *Xho* I and *Nhe* I. The bands running just below the 1 kb marker are syntaxin 4.

UC indicates lanes containing uncut plasmid while C indicates lanes containing cut plasmid. The positions of the Promega 1 kb DNA ladder are indicated.

4.3.3.B Homologous recombination of pFastBac™ Dual and the baculovirus backbone vector in E. coli

Homologous recombination

The homologous recombination between pFastBac™ Dual-S23 /S4 and the baculoviral bacmid was achieved using the *E. coli* strain DH10 Bac™. This *E. coli* strain contains the baculovirus shuttle vector termed the bacmid, which contains a mini-attTn7 target site for recombination with pFastBac™ Dual, and a helper plasmid, which provides transposition proteins to facilitate the recombination. During recombination a new plasmid is produced with the expression cassette from pFastBac™ Dual inserted into the baculovirus genome. As a positive control for subsequent transfection into Sf9 cells a recombination reaction was also set up with the +ve control pFastBac™ Dual-GUS/CAT plasmid supplied by Invitrogen. Homologous recombination was performed as outlined in section 2.7.1.

Purification of recombinant DNA

In order to screen the recombinants to verify successful recombination the bacmid from each colony selected had to be purified. Since the bacmid alone is 136 kb it was necessary to use precipitation to isolate the DNA, this is outlined in section 2.7.2.

Screening of recombinants

Since the recombinant DNA is so large it was advised to screen for positive recombinants using PCR with primers that flank the Tn7R points of recombination between the bacmid and the expression cassette. If no recombination has taken place a product of roughly 300 bp should be amplified. If recombination has occurred between the bacmid and pFastBac™ Dual alone a product of 2560 bp (the size of the expression cassette) will be amplified. Upon recombination with pFastBac™ Dual containing other gene inserts the product will be 2560 bp plus the size of the relevant genes. Bacmid recombined with pFastBac™ Dual-Gus/CAT should give an amplification product of roughly 5340 bp.

The Protocol used for the PCR screen of recombinants was adapted from the Bac-to-Bac manual. Since the expected PCR product was above 4 kb the use of platinum *Taq* polymerise was advised. The PCR screen was initially carried using the conditions as directed by the manual with the M13 forward (-40) and M13 reverse primers. However,

this was unsuccessful and, different primers and, conditions were subsequently advised by Invitrogen technical services. The primers advised were 5'CCCAGTCACGACGTTGTAAAACG 3' and 5'AGCGGATAACAATTTCACACAGG 3'.

10 µl of each PCR reaction was analysed on a 1% agarose gel, shown in Figure 4.6, as outlined in section 2.3.3.

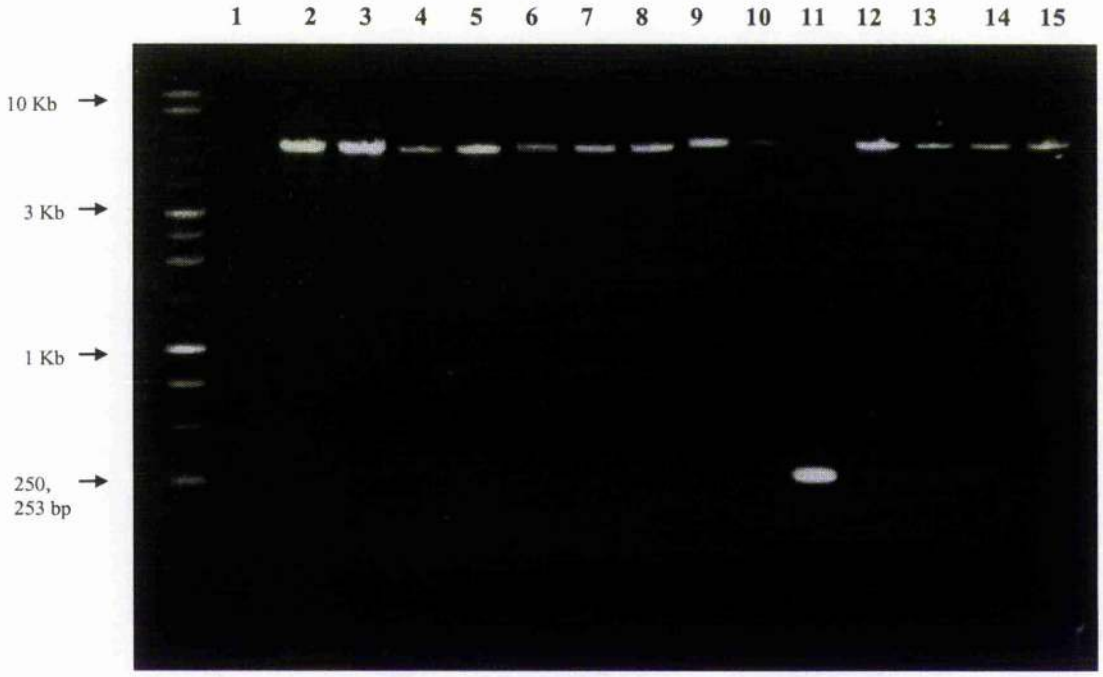


Figure 4.6: Screening of recombinants for insert using PCR

PCRs were set up as outlined above to screen for the recombination of pFastBac™ Dual-S23/S4 and pFastBac Dual™-GUS/CAT with the baculoviral genome. An aliquot of the PCR, 10 µl, was run on a 1 % agarose gel as outlined in section 2.3.3 for analysis. Lane 1 = -ve control (no template), lanes 2 - 8 = colonies from recombination of pFastBac™ Dual-S23/S4 with bacmid, lanes 9 -15 = colonies from recombination of pFastBac™ Dual-Gus/CAT with bacmid. The positions of the Promega 1 kb DNA ladder are shown.

As can be seen in Figure 4.6 the recombination of pFastBac™ Dual S4/S23 with the baculoviral genome in *E. coli* cells was successful for all colonies screened. The recombination of pFastBac™ Dual-GUS/CAT with the baculoviral genome in *E. coli* was successful for 6 out of the 7 colonies selected (clone 11 was bacmid with no recombination).Since the recombination had been successful the next step was to transfect these recombined plasmids into Sf9 cells in order to produce baculoviral particles for subsequent infections for expression of the proteins of interest.

4.3.3.C Production of baculovirus particles in Sf9 cells

Transfections of the recombinant bacmid into Sf9 cells were set up as outlined in the Bac-to-Bac manual, outlined in section 2.7.3, in order to produce the P1 virus. Initially one positive recombinant for pFastBac™ Dual-S23/S4 was chosen for transfection into Sf9 cells in order to produce a low titre P1 stock. Following initial failure to detect recombinant protein production through western blotting of the transfected cell population it was decided to transfect all positive recombinants into Sf9 cells and screen more of the positive recombinants for protein production.

4.3.4 Screening of recombinants for protein production

The pFastBac Dual-Gus/Cat bacmid was used as a positive control in the transfection and expression experiments. This expresses a gene encoding β-glucoronasidase which can be assayed for. Briefly 5 µl of 20 mg/ml X-glucoronide solution in DMSO was mixed with 50 µl of supernatant from the well transfected with this bacmid and assessed for the development of blue colour over two hours. Blue colour was established within half an hour indicating that the gene for β-glucoronasidase was being expressed and that transfection and virus production had been successful.

Following the collection of the P1 stock from each well the cells from the transfection were washed twice using PBS and subsequently lysed directly in 1.5 ml of 1X SDS-PAGE sample buffer. The lysed cells were then boiled for five minutes and 2 µl of each sample was run on a 12 % SDS-PAGE gel as outlined in section 2.2.1. The gel was then transferred and blotted against using antibodies directed against syntaxin 4 and SNAP23 as outlined in sections 2.2.2 and 2.2.3.

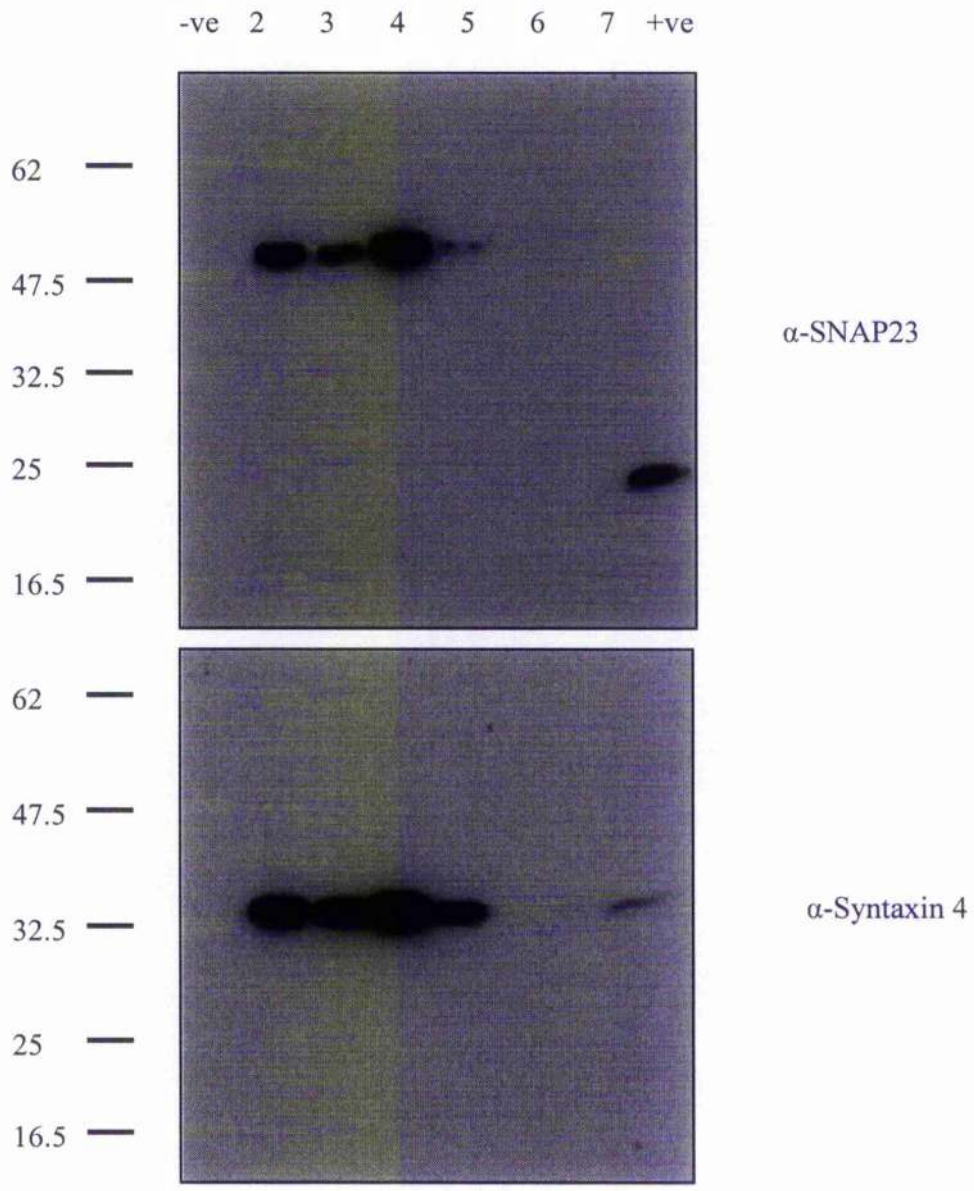


Figure 4.7: Screening of pFastBac™ Dual –S23/S4 recombinants for protein production in Sf9 cells

Recombinants were transfected into Sf9 cells using cellfectin. The cells were then incubated at 27 °C for 72 hours after which the supernatant was removed. Cells were washed two times in PBS and then lysed directly into 1X SDS-PAGE buffer. Lysed cells were boiled for 5 minutes and the lysates then run on a 12 % SDS-PAGE gel as outlined in section 2.2.1. The gel was transferred and probed for SNAP23 and syntaxin 4 as outlined in sections 2.2.2 and 2.2.3. Lane 1: -ve control (no plasmid added to transfection mixture), Lane 2-7: Recombinants from PCR screen, Lane 8: +ve control (lysate from 3T3-L1 adipocytes). The positions of the broad range markers are indicated.

The negative control indicates that neither antibody cross reacts with endogenous Sf9 protein. Cells transfected with recombinants 2-4 gave good expression of both GST-SNAP23 and syntaxin 4, which ran at the expected sizes, in Sf9 cells. Cells transfected with recombinant 5 gave good expression of syntaxin 4 but poor expression of GST-SNAP23. Cells transfected with recombinants 6 and 7 appear to have no expression of either GST-SNAP23 or syntaxin 4.

On the basis of this screen for protein expression it was chosen to amplify the supernatant from Sf9 cells transfected with recombinant 4 for subsequent expression experiments. Prior to amplification and optimisation of expression the palmitoylation status of SNAP23 expressed by the recombinant baculovirus was studied.

4.3.5 *Palmitoylation of recombinant SNAP23*

As previously mentioned *E. coli*, as prokaryotic cells, are incapable of carrying out post-translational modifications that would normally occur in mammalian cells including palmitoylation. Insect cells are eukaryotic cells and have been shown to be capable of modifying recombinant proteins post-translationally including the addition of palmitate groups.

Palmitoylated SNAP23 should be less mobile on SDS-PAGE than its unpalmitoylated counterpart (Voldez-Taubes and Pelham, 2005), due to the increase in molecular weight from the covalently attached palmitate group(s). In order to see if there was any difference in mobility between GST-SNAP23 expressed in *E. coli* and that expressed in insect cells a fraction of lysate from both cells was boiled in 2X SDS-PAGE buffer containing 10 mM DTT and run on a 8 % SDS-PAGE gel as outlined in section 2.2.1, transferred onto nitrocellulose as outlined in section 2.2.2 and blotted against SNAP23 as outlined in section 2.2.3. Data from this experiment is shown in Figure 4.8.

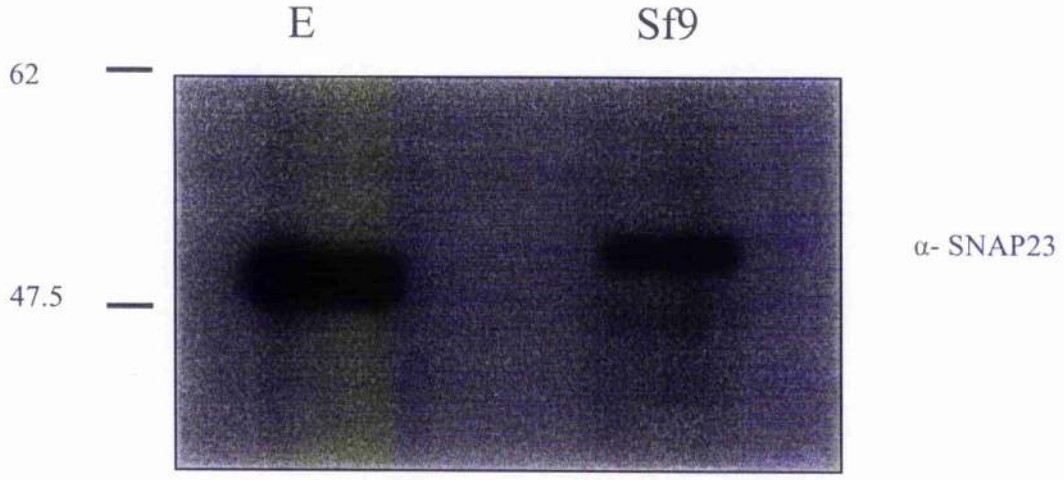


Figure 4.8: Mobility of GST-SNAP23 produced in E. coli and Sf9 cells

Cells from *E. coli* transformed with pET41a SNAP23 (shown in Lane E) or Sf9 cells infected with baculovirus expressing GST-SNAP23 (shown in Lane Sf9) were lysed in 2X SDS-PAGE sample buffer containing 10 mM DTT, boiled for 5 minutes and run on an 8 % SDS-PAGE gel as outlined in section 2.2.1. The gel was then transferred as outlined in section 2.2.2 and probed for SNAP23 with an anti-SNAP23 antibody as outlined in section 2.2.4. Positions of broad range molecular markers are shown.

As can be seen in Figure 4.8 GST-SNAP23 expressed in Sf9 cells has reduced mobility compared to that expressed in *E. coli*. This reduced mobility is most likely due to a post-translational modification that occurs only in the Sf9 cells, since the sequence encoding the fusion protein under the control of the promoter is exactly the same in each cell type. In order to establish whether this reduced mobility is due to the addition of palmitate groups to SNAP23 in Sf9 cells two techniques were employed.

Initially cell lysate from Sf9 cells, infected with the baculovirus for 72 hours at 27 °C, expressing GST-SNAP23, was treated overnight at room temperature with either with 1 M Tris pH 7.4, as a control, or 1M Hydroxylamaine pH 7.4, which is known to cleave the thioester bond which covalently attaches palmitate groups to proteins (Pepperberg *et al.*, 1995). The following morning these lysates were boiled for 5 minutes in 2X SDS-PAGE buffer and run on an 8 % SDS-PAGE gel as outlined in section 2.2.1. The gel was then transferred as outlined in section 2.2.2, and probed for SNAP23 with an anti-SNAP23 antibody as outlined in section 2.2.3. As shown in Figure 4.9A, GST-SNAP23 in the lysate treated with hydroxylamine does have increased mobility on the SDS-PAGE gel compared to that treated with Tris as a control, suggesting the difference in mobility is due to the addition of palmitate groups in Sf9 cells. The smearing in the Tris control lane may be due to degradation of the protein due to the overnight incubation at room temperature.

A second technique was used to study the palmitoylation state of GST-SNAP23 expressed in Sf9 cells. This technique was developed by Drisdel and Green (Drisdel and Green, 2004) and has been used to study the palmitoylation of yeast SNARE proteins (Valdez-Taubas and Pelham, 2005). The protocol followed was adapted from a recent publication by Valdez-Taubes and Pelham (Valdez-Taubas and Pelham, 2005). Unpalmitoylated cysteine groups are first blocked by the addition of N-ethylmaleimide (NEM) which hydroxylamine cannot cleave. Hydroxylamine is then used to cleave the thioester bond between any palmitate groups and protein. Free sulfhydryl groups of previously palmitoylated cysteines revealed by hydroxylamine cleavage can then be labelled using sulfhydryl -biotin-conjugated -specific reagent which can be pulled down using streptavidin (which binds to biotin) linked to beads. To control for non-specific binding between the protein of interest and the streptavidin beads, a pull down is run concurrently with lysate treated with NEM then Tris instead of hydroxylamine, which should not lead to any free cysteines. Pull downs were carried out as outlined in section 2.8.

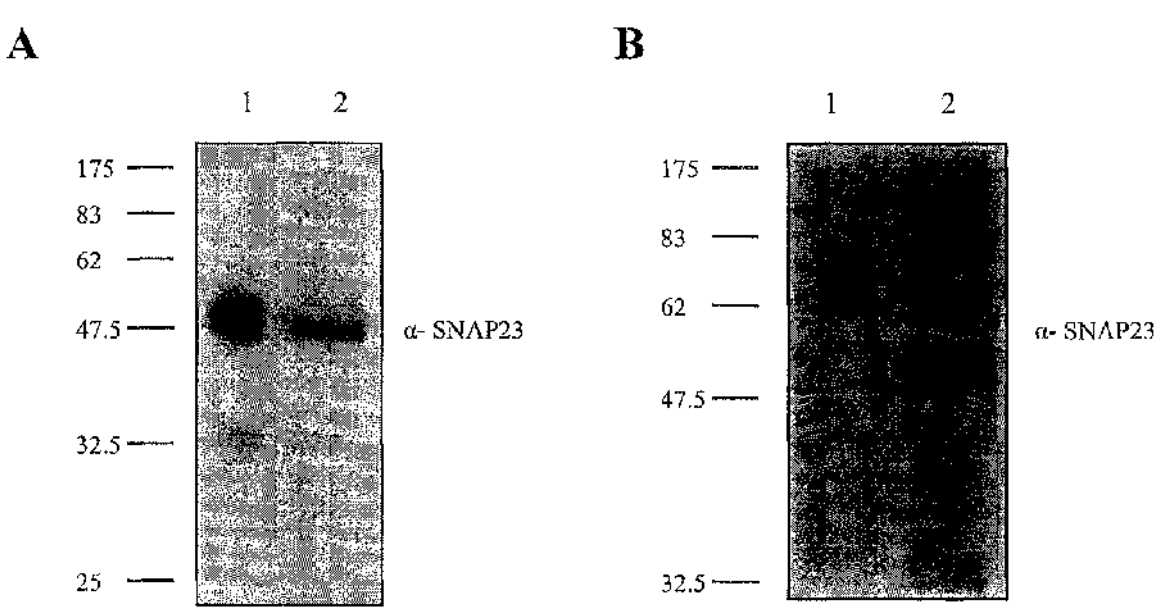


Figure 4.9: Palmitoylation of SNAP23 expressed in Sf9 cells

(A) HA treatment of Sf9 lysate. Sf9 cells were infected with recombinant baculovirus for 72 hours. Lysate was prepared in A200 with the addition of 1 % OG, 2 mM PMSF and complete protease inhibitors. Cells were broken by ten passes through a 10 gauge needle. Equal volumes of lysate were incubated with an equal volume of 1 M Tris pH7.4 (Lane 1, as a control) or 1 M hydroxylamine pH7.4 (Lane 2) overnight. The lysate was then run on a 8 % SDS-PAGE gel and blotted for SNAP23 as outlined in sections 2.2.1, 2.2.2 and 2.2.3 **(B)** Streptavidin-agarose pull down from Sf9 lysate, incubated with 25 mM NEM, treated with either 1M Tris pH7.4 (Lane 1) or 1 M hydroxylamine pH7.4 (Lane 2) and 300 μM biotin-BMCC. The positions of the broad range markers are indicated.

As can be seen in Figure 4.9B GST-SNAP23 is only pulled down by streptavidin-agarose from lysate treated with hydroxylamine confirming that GST-SNAP23 expressed in Sf9 cells infected with recombinant baculovirus is palmitoylated.

As it had been established that SNAP23 expressed by the baculovirus in Sf9 cells was palmitoylated, the baculovirus was amplified and used for expression and purification studies.

4.3.6 Amplification of initial low titre stock

Since the initial P1 stock is of very low titre, it is necessary to amplify the virus for large scale protein expression. To amplify this initial P1 stock, the amplification protocol supplied on the website for the institute of cancer research structural biology was followed. Briefly, Sf9 cells were seeded in Sf-900II at a density of 2x 10^6 cells/ml in 15 ml per 150 cm^2 flask. Each flask was inoculated with 300 µl of P1 stock and cultured at 27 °C for 72 hours. At the end of this incubation the supernatant was removed and spun at 500 xg to clear any cell debris. The baculovirus P2 stock was stored at 4 °C.

4.3.7 Optimisation of expression of SNAREs using the recombinant baculovirus

In order to optimise the expression of the two target proteins before progressing to large-scale protein expression, two factors, dose response and time course were considered. Cells were seeded into a 24 well plate at a density of 6 x 10^5 cells per well and allowed to attach for 1 hour. The media was removed and the cells were washed once with Sf-900II medium. After washing, 300 µl of Sf-900 II medium was placed into each well. The baculovirus P2 stock was assumed to have a titre of 1 X 10^7 pfu/ml as multiple attempts to quantify the titre using plaque assay were unsuccessful. The baculovirus stock was then added to each well at the desired dose (M.O.I. was calculated as set out in the Bac-to-Bac manual). The cells were incubated at 27 °C for the appropriate amount of time after which cells were washed twice with 300 µl PBS and lysed directly into 400 µl of 1 x SDS-PAGE sample buffer. The samples were frozen at –20 °C until all samples could be boiled for 3 minutes, run on a 12 % SDS-PAGE gel and transferred in order to blot for the relevant proteins as outlined in section 2.2.1, 2.2.2 and 2.2.3. Data is shown in Figure 4.10.

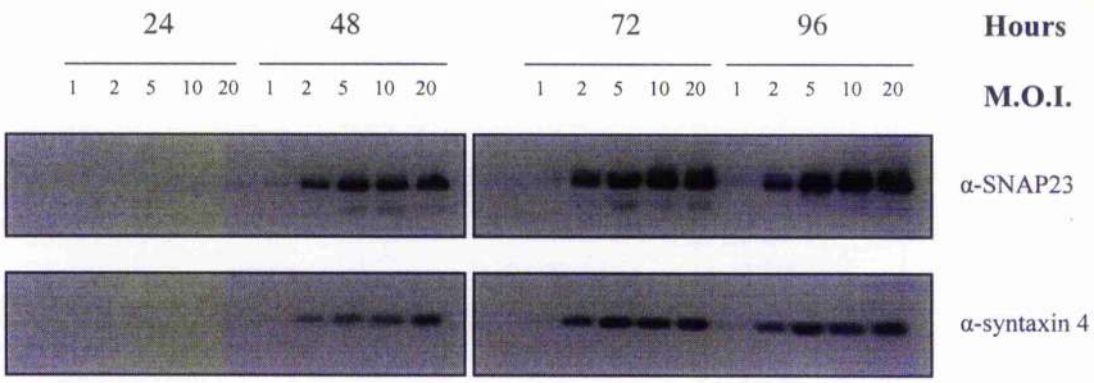


Figure 4.10: Optimisation of expression of syntaxin 4 and SNAP23 using recombinant baculovirus

Cells seeded at a density of 6 x 10^5 cells per well in a 24 well plate in 300 µl of Sf-900 II were infected with a set M.O.I. of baculovirus and cultured for the indicated period of time at 27 °C. After the indicated time cells were washed twice with 300 µl PBS and lysed directly into 400 µl of 1 x SDS-PAGE sample buffer. The samples were frozen at –20 °C until all samples could be boiled for 3 minutes. Samples were run on a 12 % SDS-PAGE gel as outlined in section 2.2.1, transferred onto nitrocellulose as indicated in section 2.2.2 and probed for SNAP23 and syntaxin 4 as outlined in section 2.2.3.

Expression of proteins under the control of very late promoters normally peaks around 48-72 hours post-infection. From Figure 4.10 it was decided to infect the Sf9 cells at an M.O.I. of 5 and culture them for 72 hours, as this gave a good expression of both of the proteins, prior to collecting the cells for subsequent protein purification.

4.3.8 *Purification of recombinant protein from Sf9 cells*

Following the optimisation of protein expression large-scale protein expression was attempted. Sf9 cells were grown to a density of 6 x 10^7 cells/ml in a total of 48 150 cm^2 each containing 30ml. The cells were inoculated with the appropriate amount of virus and allowed to grow for 72 hours at 27 °C. The media was removed from the flasks and the cells were gently washed twice with PBS. The cells were then dislodged in 50 ml of lysis buffer (A200 containing 4 % OG, 2 mM PMSF and complete protease inhibitors) by rapping the flasks, and resuspended cells were frozen at –80 °C. The resuspended cells were thawed on ice, and broken by 2 passes through the French press at 950 psi. Insoluble material was pelleted by centrifugation at 30,000 xg in a JA20 rotor for 60 minutes, and the GST-tagged protein in the supernatant was bound to 2.5 ml of glutathione sepharose pre-equilibrated with lysis buffer overnight on a rotator in the cold room. The following day the beads were washed in 7 times with 20 ml of A200 containing 1 % OG, and resuspended in 1 ml of the same buffer to which 100 units of thrombin had been added. Thrombin cleavage, was carried out at room temperature for 4 hours on a rotator. Following thrombin cleavage the supernatant containing the cleaved protein was collected from beads that had been centrifuged for 5 minutes at 500 xg. Aliquots of washes, beads, cleaved products and supernatant (15 µl) were boiled in 2X SDS-PAGE sample buffer for 5 minutes and analysed for protein content by running on an 12 % SDS-PAGE gel as outlined in section 2.2.1 which was stained using Coomassie blue as outlined in section 2.2.4. The data are shown in Figure 4.11

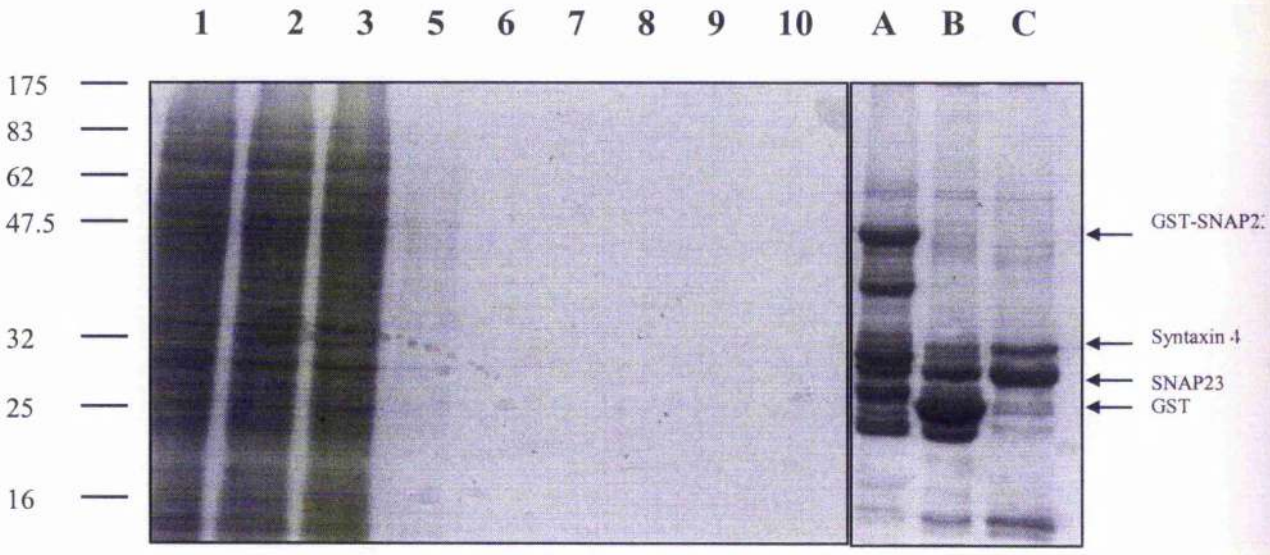


Figure 4.11: Purification of syntaxin 4/SNAP23 complex from Sf9 cells

Sf9 cells were infected with recombinant baculovirus, and syntaxin 4/SNAP23 was purified by virtue of the GST tag on SNAP23. The cells were washed once with PBS and resuspended in A200, with the addition of 4 % OG, 2 mM PMSF and complete protease inhibitors. Cells were lysed by two passes through a French press at 950 psi and insoluble matter pelleted by spinning at 30,000 xg in a JA20. Lane 1 shows the cell lysate pre-spin and lane 2 shows the lysate post-spin. The lysate was then bound overnight to glutathione sepharose. The beads were washed 7 times with 20 ml of A200 containing 2 mM DTT and 1% OG, an aliquot (15 µl) of each wash is shown in lanes 3-10. The protein bound to beads, shown in lane A (15 µl), was then liberated from the beads by incubation with thrombin for 4 hours at room temperature. Lane B (15 µl) contains the cleaved products and lane C (15 µl) contains the soluble supernatant collected from the beads. Samples were boiled for 5 minutes in 2X SDS-PAGE buffer and run on a 12 % SDS-PAGE gel as outlined in 2.2.1 and stained with Coomassie blue as outlined in section 2.2.4. The positions of the broad range markers are indicated.

Figure 4.11 shows the purification of syntaxin 4/SNAP23 complex from Sf9 cells infected with recombinant baculovirus. The pre and post spin lanes show very similar levels of total protein indicating that the majority of protein expressed in Sf9 cells was soluble in the lysis buffer. In the lane containing the supernatant cleaved from the beads two bands of the expected size for the products of syntaxin 4 and SNAP23 can clearly be seen. The t-SNARE complex of syntaxin 4/SNAP23 can therefore be successfully purified from Sf9 cells infected with recombinant baculovirus.

4.4 Discussion

In this chapter I have investigated the influence of sphingomyelin and cholesterol on fusion facilitated by the SNARE proteins known to be involved in Glut4 vesicle exocytosis by incorporating these lipids into t-SNARE liposomes used for the *in vitro* fusion assay set up in Chapter 3. I have also successfully engineered a baculovirus to express the t-SNARE complex of syntaxin 4 and SNAP23 in order to purify this complex from a eukaryotic host in which SNAP23 should hopefully be palmitoylated.

SNAREs have been localised to lipid rafts, isolated as DRMs, in many cell types. The function of these domains in the regulation of SNARE mediated fusion is yet to be established. According to the zipper model of SNARE mediated fusion, the SNARE complex zippers together from the N to the C terminus bringing the membranes into close apposition and facilitating fusion. Studies in yeast and *in vitro* fusion data have both emphasised the importance of the transmembrane domain of SNAREs in fusion (Grote *et al.*, 2000;McNew *et al.*, 2000b). The replacement of the transmembrane domain of synaptic SNAREs with lipid anchors that did not span the lipid bilayer allowed formation of the core complex, *in vitro*, but prevented fusion of the membranes (McNew *et al.* 2000b). When these lipid anchors were replaced with longer lipids which could span the bilayer *in vitro* fusion was facilitated (McNew *et al.*, 2000b). This suggests that force must be transduced through the membrane to facilitate fusion and, merely bringing the membranes into close apposition is not sufficient. Within the cell the same principle appears to be true as replacing the transmembrane domains of yeast exocytic SNAREs with geranyl-geranyl moieties (C16) leads to a block in fusion (Grote *et al.*, 2000). The study of fusion between large unilammelar vesicles with varying lipid compositions *in vitro* has also supported a role for lipid composition in the regulation of fusion (Brock *et al.*, 1994). The *in vitro* fusion of liposomes to an intact secretory granular fraction from rat parotid glands has also been shown to be dependant on the lipid composition of the liposomes (Mizuno-kamiya *et al.*, 1995). In *S. cerevisiae* membrane composition has been shown to influence the ability of SNAREs to facilitate membrane fusion (Colucciois *et al.*, 2004). Recently it has shown that the acyl-CoA family of lipids, which have an inverted cone shape, inhibit fusion in the *in vitro* fusion facilitated by SNAREs (Melia *et al.*, 2006). It is therefore possible that the lipid composition surrounding the transmembrane domains of the SNARE proteins influences fusion. Recruitment of

SNAREs into lipid raft domains may have a direct influence on fusion facilitated by SNARE proteins due to the different geometries of the resident lipids.

Although the fusion assay showed that there was little difference in the rounds of fusion supported by vesicles with either the standard lipid composition, the addition of 33 mol% cholesterol or 20 mol% sphingomyelin/33 mol% cholesterol this data can unfortunately not be fully interpreted due to a substantial difference in liposome diameter on incorporation of these lipids. The calculation to determine rounds of fusion assumes the two fusing populations of liposomes are of approximately equal size. The difference in the diameter of the t-SNARE liposomes means that the data from the 3 different liposome populations cannot be directly compared. In order to directly compare this fusion data new calibration curves would have to be produced using the different proportions of lipids that each round of fusion would incur for each of the different liposome population, this was unfortunately out with the time constraints of this project.

The majority of *in vitro* studies on SNARE proteins have been carried out using SNAREs expressed and purified from *E. coli*. Although these SNAREs support fusion *in vitro*, the rate of fusion is much lower than that observed *in vivo*. This is possibly due to the lack of additional co-factors. This could also possibly be due to the absence of palmitate groups attached to SNAP25 and SNAP23 purified from this prokaryotic host. Recently it has been shown that SNAP25 expressed by baculovirus in insect cells is palmitoylated (Kammer *et al.*, 2003). In order to express and purify the t-SNARE complex, syntaxin 4 and SNAP23, from insect cells a baculovirus expressing both of these genes was engineered. This was successfully constructed to express both full-length syntaxin 4 and SNAP23 simultaneously. Analysis of SNAP23 revealed that like SNAP25, this protein is also palmitoylated within insect cells. Although these data showed that SNAP23 expressed in Sf9 cells is palmitoylated, it cannot be established from these techniques alone how many palmitate groups have been added to the protein, or the location of the cysteine residues that are modified by palmitate. In order to establish this, mutational analysis of the cysteine residues in the linker domain of SNAP23, and quantification of either streptavidin-pull downs or ^{3}H palmitate labelling would have to be carried out. It is possible that the acylation of SNAP23 on the central cysteine region has a direct affect on fusion, independent of its influence on targeting this protein to raft domains. In order to compare the ability of palmitoylated SNAP23 and unpalmitoylated SNAP23 to facilitate fusion, *in vitro* fusion assays using liposomes reconstituted with t-SNARE complex

purified from *E. coli* and Sf9 cells could be carried out. Unfortunately this was out with the time constraints of this study.

Chapter 5

Conformation of syntaxin 4 and interaction with Munc 18c

5.1 Introduction

A family of proteins called SNAREs mediates the fusion of intracellular membranes. Although it has been demonstrated that SNAREs are necessary and sufficient to facilitate this fusion *in vitro,* it is clear that this event is regulated by other proteins *in vivo*. One family of proteins known to be particularly important in this regulation is the Sec1/Munc 18 (SM) family of proteins. Although, like SNAREs, the SM family of proteins are highly conserved and are known to play a critical role in membrane traffic, their precise role is yet to be established. Investigation into the conformation of syntaxins and their interaction with SM proteins has revealed two main modes of binding between these families of proteins.

5.1.1 Structural studies of SNARE complexes

The biophysical characterisation of bacterially produced SNARE proteins in monomeric and complexed forms has yielded important information about their conformations.

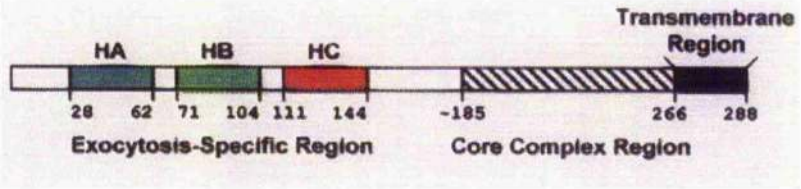
The first syntaxin to be identified, and by far the most thoroughly characterised, is the neuronal plasma membrane syntaxin 1a. In addition to the SNARE domain, this syntaxin has a large N-terminal domain that comprises roughly two thirds of the full-length protein sequence (Fernandez *et al.*, 1998). The general structure of the N-terminal domain of syntaxin family members consists of a short N-terminal peptide followed by 3 helices termed Ha, Hb and Hc (Fernandez *et al.*, 1998), as shown in Figure 5.1A. Analysis of the N-terminal domain of syntaxin 1a by NMR and circular dichroism (CD) has shown that these three helices interact, and are found folded into a three helical bundle termed the Habc domain as shown in Figure 5.1B (Fernandez *et al.*, 1998). In syntaxin 1a the Habc domain encompasses residues 28-144 (Fernandez *et al.*, 1998). Residues 1-27, the N-terminal peptide, and 145-180, from the end of the Hc helix to the SNARE domain comprising the linker region were found to be unfolded (Fernandez *et al.*, 1998). The use of electron paramagnetic resonance spectroscopy has predicted this linker region to be highly flexible and capable of undergoing conformational change (Margittai *et al.*, 2003).

NMR data also highlighted the existence of a groove between the interface of helix b and c that is postulated to be important in binding to effector molecules or the SNARE domain (Fernandez *et al.*, 1998). A subsequent study using X-ray crystallography of the Habc

domain showed that this groove contains a high proportion of hydrophilic residues strengthening the probability that this domain folds back on the SNARE domain (Lerman *et al.*, 2000).

The overall domain structure of syntaxin 1a, as depicted in Figure 5.1A, is conserved through all syntaxins characterised so far although the exact length of the N-terminal domain does show some variation. The sequence of the N-terminal domain of plasma membrane syntaxins (syntaxin 1, 2, 3, 4 and yeast isoforms Sso1p and Sso2p) involved in exocytosis show a high degree of conservation that is not observed in other syntaxins (Fernandez *et al.*, 1998). Thus it is possible that the N terminus of plasma membrane syntaxins may play a specific role in regulating the specialised form of fusion exocytosis.

A



B

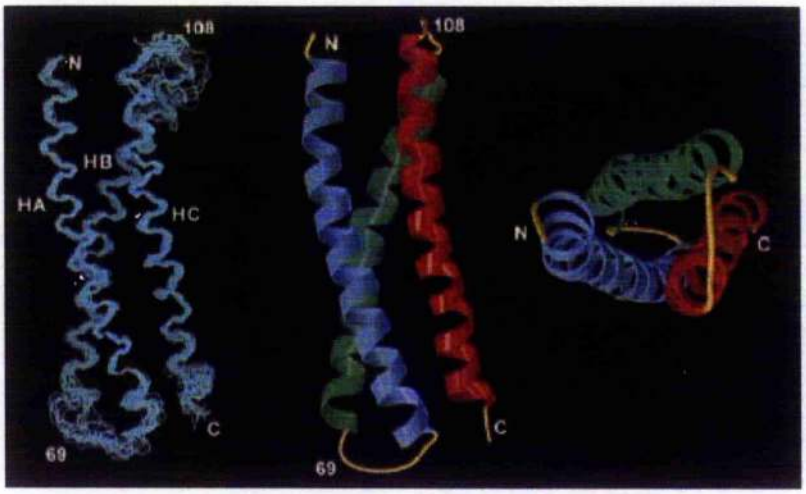


Figure 5.1: General structure of the syntaxin family

(A) Plasma membrane syntaxins contain a C-terminal transmembrane region, a C-terminal conserved core complex region (the SNARE domain which is involved in forming the core complex) joined through a flexible linker domain to the N-terminal domain composed of three helices termed Ha, Hb and Hc, collectively known as the Habc domain. This N-terminal domain is preceded by a short N-terminal unstructured peptide. (B) The Habc domain viewed in 2 different orientations. The Ha, b, c helices form a 3 helical bundle. Residues annotated are representative of syntaxin 1A (Fernandez *et al.*, 1998)

5.1.2 Conformation of syntaxins

Members of the syntaxin family have been shown to exist in two distinct conformations (Hanson *et al.*, 2000). In the so-called "open" conformation the Habc domain is spatially separated from the SNARE motif, which is therefore available to participate in SNARE complexes (Toonen and Verhage, 2003). In the "closed" conformation, the Habc domain folds down and shields the SNARE motif rendering it inaccessible for entry into SNARE complexes (Toonen and Verhage, 2003).

Different syntaxins seem to adopt either a "closed" or "open" conformation in solution, and the conformation they adopt appears to influence the mode through which SM proteins can interact with them.

5.1.2.1 Closed syntaxins and binding of SM proteins

In syntaxin 1a and its yeast homologue Sso1p, the N terminus has been shown to fold back on the SNARE domain holding these proteins in a so-called closed conformation.

Munc 18A/syntaxin 1a

Surface plasmon resonance studies of syntaxin 1a have shown that the Habc domain binds weakly to the SNARE domain (Calakos *et al.*, 1994). This binding of the Habc domain to the SNARE domain inhibits the binding of both synaptobrevin (Calakos *et al.*, 1994;Pevsner *et al.*, 1994a) and SNAP25 (Pevsner *et al.*, 1994a). In the SNARE complex the N-terminal domain can be seen flexibly linked to the core complex by EM (Hanson *et al.*, 1997). Thus it appears that syntaxin 1a can exist in two conformations, referred to as "open" when in complex with other SNAREs and "closed" in isolation where the N-terminal domain folds back onto the SNARE motif. This closed conformation was also observed using NMR of monomeric recombinant syntaxin 1a (Dulubova *et al.*, 1999). Mutations engineered into syntaxin 1A, L165A and E166A, were found to prevent the formation of the closed conformation and hold the molecule in a constitutively open form in isolation (Dulubova *et al.*, 1999). These mutations are within a postulated helix in the linker region (Dulubova *et al.*, 1999), which is thought to contact both the SNARE domain and the Hc helix (Margittai *et al.*, 2003). While the SM protein Munc 18a was found to

bind with high affinity to wild type syntaxin 1a the open form was found to be incapable of binding to Munc 18a (Dulubova *et al.*, 1999).

Crystallographic studies have revealed that Munc 18a is an arched shaped molecule with a central cavity that cradles monomeric syntaxin 1a in its closed conformation making contact with syntaxin 1a through domains 1 and 3, as shown in Figure 5.2 (Misura *et al.*, 2000b). Interestingly the structure adopted by syntaxin 1a in this complex is similar to the structure of monomeric syntaxin 1a (Dulubova *et al.*, 1999). This suggests that Munc 18a acts to hold syntaxin 1a in a closed conformation. Consistent with this observation it has been reported that Munc 18a does not bind to the binary complex of syntaxin 1a/SNAP25 or the ternary complex of syntaxin 1a/SNAP25/VAMP *in vitro* (Yang *et al.*, 2000). This highlights the possibility of an inhibitory influence of this interaction.

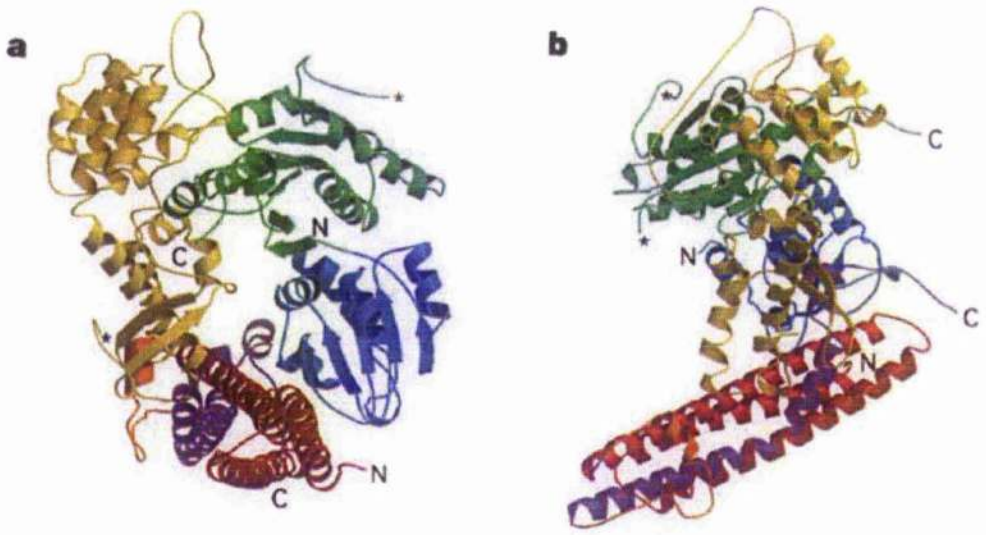


Figure 5.2: Crystal structure of Munc 18a interaction with syntaxin 1a

Two different views of the crystal structure of Munc 18a in complex with syntaxin 1a. Domain 1 of Munc 18a is shown in blue, domain 2 in green and domain 3 in yellow. The H3 domain of syntaxin 1a is depicted in purple, the Habc/H3 linker in orange and the Habc domain in red. Munc 18a forms an arched shape structure which cradles syntaxin 1a in its closed conformation (taken from Misura *et al.*, 2000b).

Sso1/Sec1p

Like syntaxin 1a, the N-terminal domain of Sso1p inhibits the binding of the SNAP25 homologous region of Sec9p to the SNARE domain (Nicholson *et al.*, 1998). In support of Sso1p adopting a default closed conformation, gel filtration assays and CD measurements demonstrated that removal of the N-terminal domain of Sso1p led to greater than 2000-fold increase in the rate of binary complex formation with the SNAP25 homologous portion of Sec9p (Nicholson *et al.*, 1998). Isolated C and N-terminal domains were also demonstrated to interact directly using a gel filtration assay, supporting the adoption of a closed conformation (Nicholson *et al.*, 1998). A subsequent study performed by Fiebig *et al.*, using NMR of the cytosolic portion of Sso1p, along with CD and size exclusion chromatography of a mixture of the Habc domain, and the Hcore domain gave complimentary data to the previous study (Fiebig *et al.*, 1999). Sso1p has since been confirmed to adopt a closed conformation using X-ray crystallography (Munson *et al.*, 2000).

However, unlike the binding of Munc 18a to syntaxin 1a it was initially shown that monomeric Sso1p, proposed to be in the closed conformation, did not bind to its cognate SM protein Sec1p and that Sec1p only bound to the assembled yeast exocytic complex (Carr *et al.*, 1999). This data has recently been called into question by a subsequent study which showed that Sec1p could bind monomeric Sso1p but with a lower affinity than the binding to the exocytic binary or ternary complex (Scott *et al.*, 2004).

These studies support a model in which SM proteins hold their cognate syntaxins in a closed conformation and thus regulate SNARE complex assembly, perhaps by facilitating the switch of syntaxins from a closed to an open form. This model however, has recently been called into question with the discovery that some syntaxins use an alternative mode of binding to their SM proteins.

5.1.2.2 Open syntaxins and binding of SM proteins

In other members of the syntaxin family, Vam3p, Sed5p, Tlg2p and Pep12p (Dulubova *et al.*, 2001;Dulubova *et al.*, 2002), the Habc domain does not appear to fold back upon the SNARE domain, and these SNAREs are thought to exist in a constitutively open form. Some of these SNAREs have a 30 amino acid peptide sequence prior to the Habc domain, which is thought to be important in the binding of their cognate SM protein.

Vam3p is a yeast syntaxin involved in vacuolar fusion. NMR studies of this syntaxin revealed that, despite no sequence homology with the N terminus of plasma membrane syntaxins, the N terminus of Vam3p folds into a three helical bundle very similar to that observed for syntaxin 1a and Sso1p (Dulubova *et al.*, 2001). However, unlike these plasma membrane syntaxins, the Habc domain of Vam3p contains no groove and was not found to fold back on the SNARE domain holding the protein in a closed conformation (Dulubova *et al.*, 2001). In addition, it was found that only the SNARE domain was necessary to facilitate binding of the appropriate SM protein Vps33p to Vam3p from yeast lysates (Dulubova *et al.*, 2001) suggesting a completely novel mode of binding observed for a SM protein to its cognate syntaxin. A direct interaction between Vps33p and Vam3p is yet to be shown.

Through a yeast two-hybrid screen and GST pull downs, the yeast SM protein Sly1 was found to bind to Sed5p (which localises to the Golgi) and Ufe1p (which localises to the ER) through a short 20 amino acid peptide present at the N terminus of these syntaxins (Yamaguchi *et al.*, 2002). NMR analysis showed that Sed5p also has a three helical folded Habc domain (Yamaguchi *et al.*, 2002). The crystal structure of the complex of Sed5p and Sly1p was solved, and revealed a 45 amino acid N-terminal peptide of Sed5p interacted with domain I of the SM protein Sly1p, a completely different mode of binding from that observed for Munc 18a and syntaxin 1a (Bracher and Weissenhorn, 2002). This result was highly unexpected since the two SM proteins have a highly similar arch shaped structure (Misura *et al.*, 2000b;Bracher and Weissenhorn, 2002). In addition to binding to monomeric Sed5p through this N-terminal interaction, Sly1 was also found to bind to the assembled SNARE complex of Sed5p/Bet1p/Bos1p/Sec22p and to regulate the specificity of which SNARE complexes Sed5p can enter into (Peng and Gallwitz, 2002). Sly1p has also been shown to bind to non-syntaxin SNAREs Bet1p, Bos1p, Sft1p and Gos1p through interactions with their SNARE motifs (Peng and Gallwitz, 2004). This discovery led to the suggestion that the SM protein serves to bridge the v- (Bet1p or Sft1p) and t-SNAREs (Bos1p or Gos1p) from separate membranes, possibly ensuring the specificity of SNARE complex assembly, or catalysing the assembly into a four helical bundle, and hence membrane fusion.

Similar to that of Sed5p, the N terminus of Tlg2p (a syntaxin involved in traffic in the TGN and early endosome) has been shown by NMR to have a structured Habc domain. However, this syntaxin does not display a closed conformation *in vitro* (Dulubova *et al.*,

2002). Like the binding of Sed5p to Sly1p, the binding of Tlg2p to Vps45p occurs through a 33 amino acid N-terminal peptide motif (Dulubova *et al.*, 2002). It has recently been suggested that Tlg2p may be capable of adopting a closed conformation *in vivo*. A chaperone role for Vps45p has been suggested since it prevents the proteosomal degradation of Tlg2p (Bryant and James, 2001). In proteosome-deficient cells Vps45p was required for assembly of the SNARE complex containing Tlg2p, Tlg1p and Vti1p, suggesting it positively influences the formation of this complex (Bryant and James, 2001). In the absence of both Vps45p and the proteasome, an N-terminal truncated version of Tlg2p, suggested by the authors to be equivalent to open, is capable of forming SNARE complexes (Bryant and James, 2001). Vps45p has therefore been suggested to facilitate a conformational change in Tlg2p that switches it from a closed conformation, which is inaccessible to participation in SNARE complexes, to an open conformation (Bryant and James, 2001). In addition to binding to monomeric Tlg2p in *vitro* (Dulubova *et al.*, 2002) Vps45p has been shown to bind to the fully assembled cis-SNARE complex of Tlg2p/Tlg1p/Vti1p/Snc2p but not trans-SNARE complexes *in vivo*, suggesting a role in the regulation of SNARE complex formation (Bryant and James, 2003). Recently, Vps45p has also been shown to bind to Snc2p (Carpp *et al.*, 2006), strengthening the hypothesis that some SM proteins may serve to bridge the gap between t- and v-SNAREs on opposing membranes.

The syntaxin Pep12p, involved in late endosome traffic, binds indirectly to Vps45p (Bryant and James, 2001). Pep12p was also shown to have a folded Habc domain, but again, this domain was not found to fold back on the SNARE domain by NMR spectroscopy (Dulubova *et al.*, 2002). The N-terminal peptide preceding the Habc domain was found to be shorter in Pep12p than in Tlg2p, may explain the fact that the SM protein Vps45p can not bind directly to this syntaxin (Dulubova *et al.*, 2002).

5.1.3 Binding modes of SM proteins to syntaxins

In summary there appears to be three distinct modes of binding of SM proteins to their cognate syntaxin:

1. The SM protein binds the closed conformation of syntaxin. This binding requires the Habc domain and the SNARE domain.

2. The SM protein binds the open form of the syntaxin. This binding involves a short peptide sequence located N-terminally to the Habc domain.

3. Indirect binding of the SM protein to the syntaxin, facilitated by interactions with other proteins.

5.1.4 Binding of Munc 18c to syntaxin 4

Immunoprecipitation experiments from COS cells (Araki *et al.*, 1997), and a yeast three-hybrid screen (Thurmond *et al.*, 1998), have shown that Munc 18c inhibits the binding of SNAP23 to syntaxin 4. A similar inhibition of the binding of SNAP25 to syntaxin 1a has been observed with Munc 18a (Pevsner *et al.*, 1994a). Munc 18c has also been shown to inhibit the binding of VAMP2 to syntaxin 4 through *in vitro* pull downs (Tellam *et al.*, 1997), and a yeast three hybrid approach (Thurmond *et al.*, 1998). This suggests that Munc 18c may hold syntaxin 4 in a closed conformation much like that seen for syntaxin 1a, and prevent syntaxin 4 from entering into SNARE complexes. Pull downs from solubilised adipocytes have shown that, although syntaxin 4 is capable of binding both SNAP23 and Munc 18c, Munc 18c is only able to bind syntaxin 4 (Tamori *et al.*, 1998), supporting the notion that Munc 18c holds syntaxin 4 in a closed conformation. This data suggests a negative role for Munc 18c in the fusion of GSVs to the plasma membrane of insulin responsive cells.

Immunoprecipitations from basal and insulin stimulated CHO/IR cells, overexpressing Munc 18c, showed that upon insulin stimulation Munc 18c dissociates from syntaxin 4 (Thurmond *et al.*, 1998). This supports a model whereby Munc 18c holds syntaxin 4 in a closed conformation, which is released upon insulin stimulation.

5.2 Aims of this chapter

Studies on the exocytic neuronal and yeast syntaxins have shown that the assembly of SNARE complexes may in part be dependent on the conformation adopted by syntaxin. Monomeric syntaxin 1a favours a closed conformation whereas as part of the SNARE complex syntaxin 1a exists in an open conformation. Mutations within the hinge region, between the SNARE domain and the Habc domain, of syntaxin 1a lead to an open conformation. The aim of this chapter was to test the hypothesis that monomeric syntaxin 4 also adopts a closed conformation and that equivalent mutations in the hinge region cause syntaxin 4 to undergo a similar change in conformation.

5.3 Methods and Results

Most structural and biophysical studies have been carried out using the cytoplasmic domains of SNAREs since the transmembrane region is dispensable for complex formation. In order to assess whether monomeric syntaxin 4 adopts the same default closed structure as syntaxin 1a the cytoplasmic domains of these proteins were expressed and purified in *E. coli* for further study. Rational mutations based on the mutations which cause monomeric syntaxin 1a to switch from the closed to the open form (Dulubova *et al.*, 1999) were introduced into the cytoplasmic domain of syntaxin 4.

5.3.1 Alignment of syntaxin 4 against syntaxin 1a

In order to design the mutations to be introduced into the cytoplasmic domain of syntaxin 4 the sequences of syntaxin 4 and syntaxin 1a were aligned as shown in Figure 5.3.

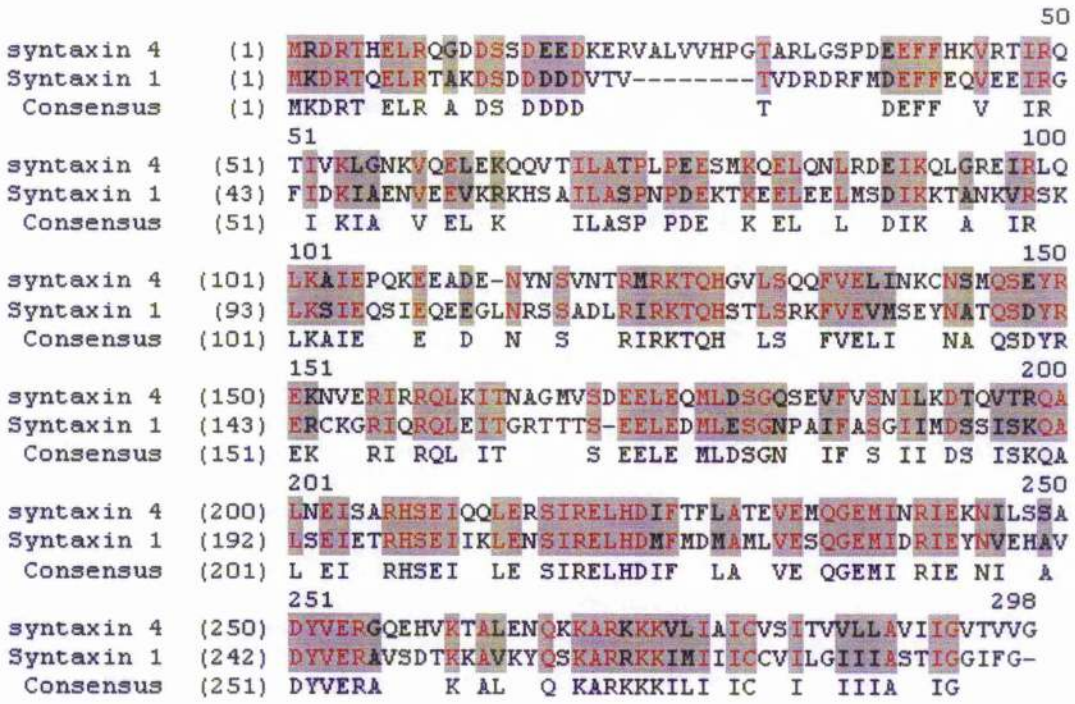


Figure 5.3: Sequence alignment of syntaxin 4 with syntaxin 1a.

Mutations to be introduced into syntaxin 4 were rationally designed by aligning the sequence of full-length syntaxin 4 with that of syntaxin 1a using Vector NTI. Residues L165/E166 in syntaxin 1a align with L173/E174 in syntaxin 4. The residues shaded in grey are the consensus between the two sequences. Vector NTI AlignX® uses the Clustal W algorithm to align multiple sequences.

5.3.2 *Site directed mutagenesis*

Mutations were rationally designed by alignment of the sequence of syntaxin 4 with that of syntaxin 1a. L173/E174 of syntaxin 4 align with L165/E166 of syntaxin 1a. It was therefore decided to mutate these residues to the same residues that had been shown to facilitate the switch of syntaxin 1a from a closed to an open state (Dulubova *et al.*, 1999).

The L173A, E174A mutations were introduced into the syntaxin 4 sequence using pGEX-syntaxin 4 cytosolic domain as a template and the QuikChange ® Site-directed mutagenesis kit. Mutations were introduced as outlined in section 2.3.2, using the primers 5'-GTGTCTGACGAGGAGGCGGCACAGATGCTGGACAGTGG-3' and 5'-CCACTGTCCAGCATCTGTGCCGCCTCCTCGTCAGACACC-3'. Once the sequence had been confirmed the plasmids were maxipreped as outlined in section 2.2.11 for long term storage at –20 °C.

5.3.3 *Purification of syntaxin 1 and syntaxin 4 cytoplasmic domains*

The cytoplasmic domains of syntaxin 1a and syntaxin 4 were expressed as GST fusions from pGEX vectors. The vectors pGEX-syntaxin 1a and pGEX-syntaxin 1a L165A/E166A were kind gifts from Robert Burgoyne (Liverpool, UK) while the pGEX syntaxin 4 plasmid was a kind gift from Jeffrey Pessin (Stony Brook, NY, USA).

The proteins were expressed in Rosetta pLysS cells as outlined in section 2.4.1. Expression was induced using 0.5 mM IPTG. Proteins were expressed at 30 °C overnight with shaking at 250 rpm. Following harvesting of the cells the cytoplasmic protein fusions were purified as outlined in section 2.4.2.

The proteins were cleaved from the GST tag using the thrombin cleavage site between the GST and syntaxin cytosolic domain coding sequences for biophysical studies. Proteins were dialysed against 4 litres of 50 mM KH_2PO_4-K_2HPO_4 pH 7.4 in the cold room overnight. Protein bound to beads and the cleaved supernatant were analysed by running on a 12 % SDS-PAGE gel followed by Coomassie Blue staining as shown in Figure 5.4.

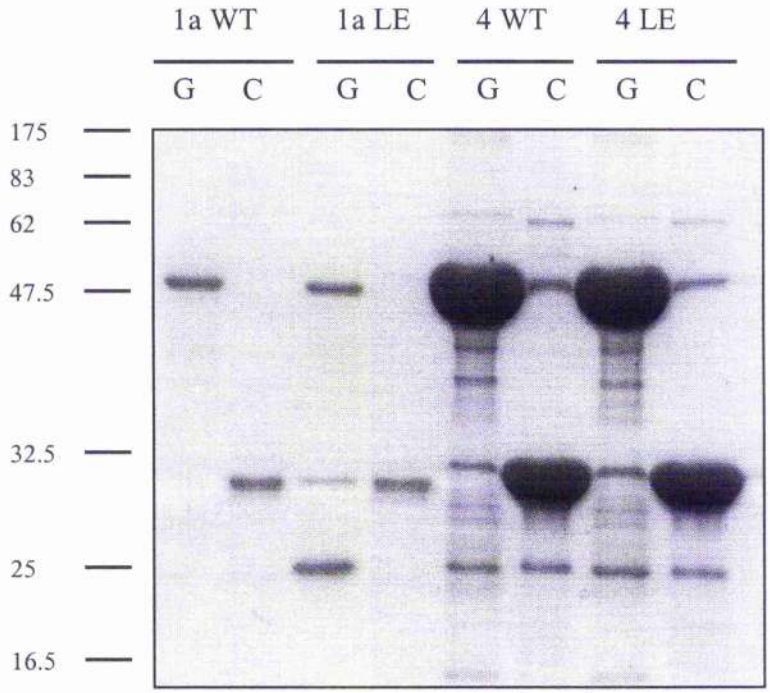


Figure 5.4: Coomassie stained gel of proteins used in this study

The cytoplasmic domains of syntaxin 1 a, syntaxin 4 and respective LE mutants were expressed in Rosetta pLysS cells as GST fusions as outlined in section 2.4.1. The proteins were purified by binding the lysate to glutathione sepharose as outlined in section 2.4.2. DNA was removed by incubation with DNase I. Stringent washes were carried out and protein bound to the glutathione sepharose was cleaved from the GST tag by 4 hours incubation at room temperature with 25 units of thrombin in PBS and collected by centrifugation at 500 xg for 5 minutes. Cleaved proteins were dialysed overnight against 50 mM KH_2PO_4- K_2HPO_4 pH 7.4. Protein bound to beads prior to thrombin cleavage (G) and dialysed supernatant (C) were run on a 12 % SDS-PAGE gel and purity analysed by staining with Coomassie blue as outlined in sections 2.2.1 and 2.2.4. The purified cytoplasmic domains were wild type syntaxin 1a (1a WT), syntaxin 1a L165A/E166A (1a LE), wild type syntaxin 4 (4 WT) and syntaxin 4 L173A/E174A (4 LE). The positions of the broad range molecular markers are shown.

As can be seen in Figure 5.4 all of the beads (lanes marked G) have one major product of the predicted size for the cytoplasmic domains of syntaxin 1a or 4 respectively fused to GST bound to them. The cleaved protein from the glutathione beads (all lanes marked C) contain only one major product, which runs at the expected molecular size for the cytoplasmic domains of syntaxin 1a or syntaxin 4 respectively. Having successfully purified the cytoplasmic domains of syntaxin 1a, syntaxin 4 and the respective LE mutants to a high degree of purity and sufficient yield biophysical experiments to study the conformation of these proteins were carried out.

5.3.4 *Circular dichroism of purified syntaxins*

Circular dichroism (CD) was used to examine and compare the secondary structures of syntaxin 1a, syntaxin 1a L165A/L166A, syntaxin 4 and syntaxin 4 L173A/E174A.

Circular dichroism is a method that is commonly used to determine the secondary structural content of proteins in solution (reviewed in Kelly and Price, 2000). It depends on the differential absorption of left and right circularly polarised light by optically active (chiral) elements within the protein. Within the protein these elements include backbone amide bonds, disulphide bonds and aromatic side chains such as tyrosine, tryptophan and phenylalanine. Within a particular secondary structure these elements will have a distinctive arrangement, which can be detected by CD.

The far UV region of the CD spectra (180 - 260 nm), which is mainly contributed by peptide bonds, gives three distinct patterns depending on the secondary structure motifs of the protein (alpha-helix, beta sheet and random coil).

The near UV region of the CD spectra (250-350 nm) is mainly contributed by the aromatic side chains and disulphide bonds and can be used to assess tertiary structure.

The composition of the buffer in which CD on proteins is carried out is critical as some buffer constituents have strong absorptions, which can mask those of the protein of interest. The CD spectra was obtained from cytoplasmic domains which had been purified and dialysed against 50 mM KH_2PO_4- K_2HPO_4 pH 7.4 in order to minimise any background absorption.

Far UV CD spectra were recorded from 180 nm to 260 nm using quartz cells of pathlength 0.05 cm, near UV CD spectra were recorded from 250 nm to 320 nm using quartz cells of 0.5 cm pavelength on a Jasco J 810 spectropolarimeter. Scanning parameters used to record the spectra were as follows · bandwidth of 1 nm, data pitch of 0.2 nm, response 0.5 seconds, scanning rate 50 nm/min and recording time point intervals of 0.25 seconds. Eight scans were collected for each protein.

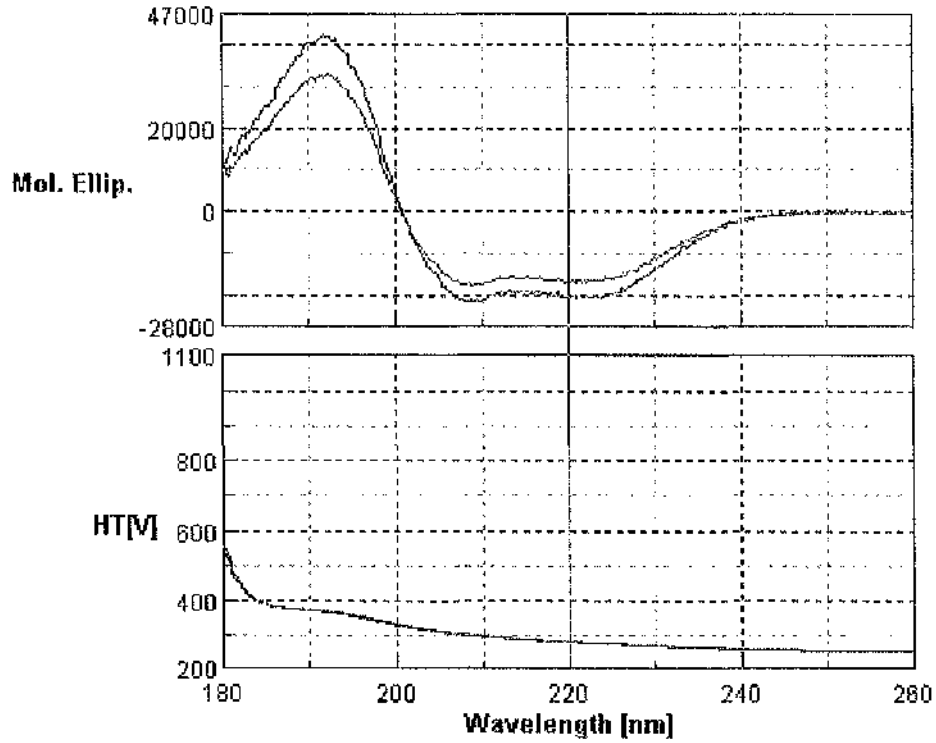


Figure 5.5: Far UV CD spectrum of the cytoplasmic domains of wild type syntaxin 1a and syntaxin 1a L165A/E166A

CD spectra was recorded for the cytoplasmic domains of wild type syntaxin 1a (Blue) and syntaxin 1a L165A/E166A (Green) from 180 to 260 nm. The proteins were diluted to 0.3 mg/ml in 50 mM KH_2PO_4- K_2HPO_4 pH 7.4. The data shown is pooled from 8 scans.

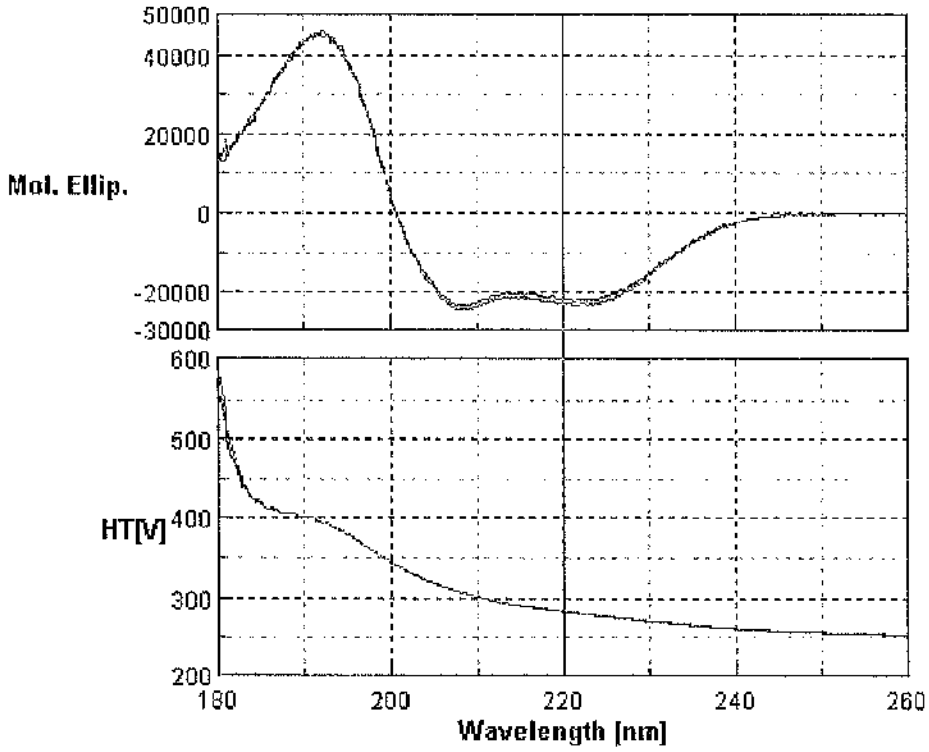


Figure 5.6: Far UV CD spectrum of the cytoplasmic domains of wild type syntaxin 4 and syntaxin 4 L173A/E174A

CD spectra was recorded for the cytoplasmic domains of wild type syntaxin 4 (Blue) and syntaxin L173A/E174A (Green) from 180 to 260 nm. The proteins were diluted to 0.3 mg/ml in 50 mM KH_2PO_4- K_2HPO_4 pH 7.4. The data shown is pooled from 8 scans.

Monomeric syntaxins are almost entirely α-helical whereas other SNARE members are unstructured in solution as assessed by CD (Fasshauer *et al.*, 1997a;Fasshauer *et al.*, 1997b). The far UV CD spectra of all 4 cytoplasmic domains show characteristic double minima of proteins with alpha helical content, as shown in Figure 5.5 and 5.6. Although there are slight differences in the far UV CD spectra of syntaxin 1a and syntaxin 1a L165A/E166A, shown in Figure 5.5, there is no significant change in overall structural content of the cytoplasmic domains from the mutations introduced into syntaxin 4, shown in Figure 5.6.

While protein spectra in the far UV region is useful for assessing the secondary structure of proteins the spectra from the near UV region (250-350 nm) is useful for studying the tertiary structure of proteins. Over these wavelengths the major contribution to the spectra is from aromatic side chains and disulfide bonds. Near UV circular dichroism requires protein concentrations in the range of 1 mg/ml, while there was a sufficient concentration of syntaxin 4 and syntaxin 4 L173A/E174A to perform this analysis there was unfortunately not enough of either syntaxin 1a or syntaxin 1a L165A/E166A to allow analysis and comparison.

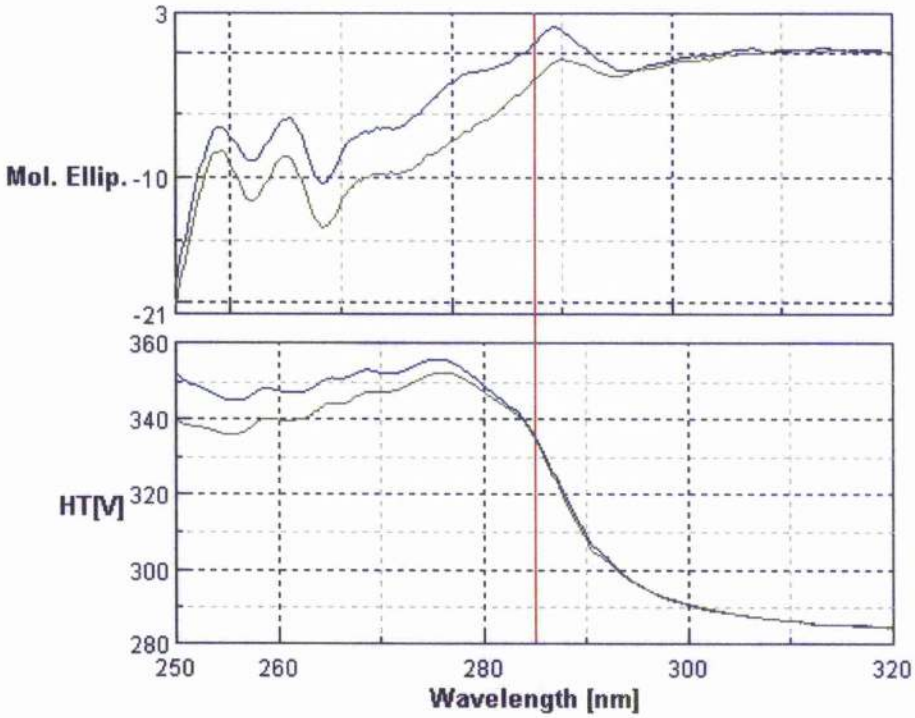


Figure 5.7: Near UV CD spectrum of wild type syntaxin 4 and syntaxin 4 L173A/E174A

Near UV CD spectra was recorded for the cytoplasmic domains of wild type syntaxin 4 (Blue) and syntaxin L173A/E174A (Green) from 250 to 320 nm. The proteins were diluted to 1 mg/ml in 50 mM KH_2PO_4- KH_2PO_4 pH 7.4. The data shown is pooled from 8 scans.

Since the main contribution of the near UV spectra is from aromatic side chains this can give information on the tertiary structure of the protein since different folding of polypeptides will lead to a variation in positions of side chains in chiral environments. From Figure 5.7 it can be seen that there is a small difference in the intensity of the peaks in the near CD region. The overall shape is similar which suggests the environments of the aromatic residues have not changed significantly. The difference perhaps suggests that the open form is less rigid.

5.3.5 *Protease digestion of syntaxin 4 and syntaxin 4 L173A/E174A*

Limited proteolysis is commonly used to assess the 3D structure of proteins (Fontana *et al.*, 1997). In general a protein folded tightly will be less susceptible to protease digestion than one folded loosely as the peptide chain will not be readily accessible to the active site of the protease. Limited proteolysis was carried out on the recombinant cytoplasmic domains of syntaxin 4 wild type and syntaxin 4 L173/E174 using chymotrypsin. Chymotrypsin cleaves peptide bonds on the carboxyl side of methionine and the aromatic side chains tryptophan, tyrosine and phenylalanine. If the WT protein does adopt a default closed conformation and the mutations in the hinge region lead to a more open form of syntaxin 4 where the Habc domain does not interact with the SNARE domain WT syntaxin should be digested at a slower rate. Syntaxin 1a L165A/E166A has been previously shown to be digested more rapidly by trypsin than wild type syntaxin 1a (Graham *et al.*, 2004).

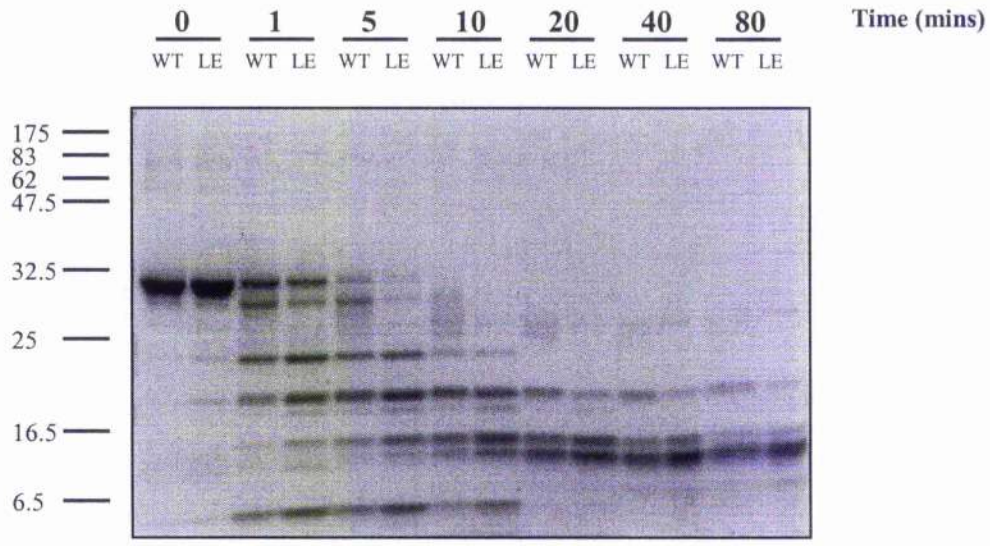


Figure 5.8: Chymotrypsin digestion of the cytoplasmic domains of wild type syntaxin 4 (WT) and syntaxin 4 L173A/E174A (LE)

Recombinant protein was diluted to 0.3 mg/ml with PBS in a total volume of 100 µl. Chymotrypsin was added at a 1:100 ratio to recombinant protein and at the time points indicated samples were taken and immediately boiled in an equal volume of 2X SDS-PAGE sample buffer, with the addition of 10 % v/v β-mercaptoethanol for 5 minutes. Samples were then stored at – 20 °C until all of the samples could be analysed by running on a 12 % SDS-PAGE gel followed by staining with Coomassie blue as outlined in sections 2.2.1. and 2.2.4. Positions of the broad range molecular weight markers are shown.

The bands correspounding to the cytoplasmic domains of syntaxin 4 and syntaxin 4 L173A/E174A were quantified by densitometry in the 0, 1 and 5 minute lanes as shown in table 5.1.

Time (mins)	% undigested wild type syntaxin 4	% undigested syntaxin 4 L173A/E174A
0	100	100
1	56	39
5	22	8.5

Table 5.1: Percentage of undigested cytoplasmic domains following treatment with Chymotrypsin

The upper band corresponding to wild type syntaxin 4 and syntaxin 4 L173A/E174A were quantified using densitometry. The quantification of each protein at time zero was set to 100 % and the results from subsequent time points were normalised to the quantification of the corresponding protein at time zero. (n=1)

As can be seen from the chymotrypsin digest in Figure 5.8 and the quantification in Table 5.1 wild type syntaxin 4 is digested at a slower rate than syntaxin 4 L173A/E174A suggesting wild type syntaxin 4 is in a more inaccessible conformation than syntaxin 4 L173A/E174A.

5.3.6 Interaction of Munc 18c with syntaxin 4 and syntaxin 4 L173A/E174A

5.3.6.1 Purification of Munc 18c

The family of SM proteins has proved difficult to express and purify from a heterologous system due to insolubility. The overexpression of protein in *E.coli* is thought to enhance the aggregation of non-native proteins due to the high level of protein crowding within the *E. coli* cell that may lead to promiscuous interactions and misfolding. Low temperatures, overnight incubations and low IPTG concentrations have all been used to increase the yield of other SM family members. The coexpression of a class of proteins called Chaperonins has been shown to increase the solubility of several recombinant proteins (Yasukawa *et al.*, 1995) including SM proteins in *E. coli* (Scott *et al.*, 2004).

Within the cells the successful folding of proteins into their unique 3 dimensional structure is facilitated by a class of proteins called chaperones (reviewed in Hourey, 2001). These chaperone proteins are thought to prevent proteins from misfolding and aggregating but not actually control the process of folding (Mogk *et al.*, 2002). During translation polypeptides emerge from the channel present in the large ribosome at a relatively slow rate and folding often occurs after the whole domain of a protein has emerged from the ribosome (Mogk *et al.*, 2002). Chaperones act to either hold the protein in a state that is competent for folding when the protein is released from the ribosome or larger chaperone proteins provide an environment for the whole protein to fold isolated from the cytosol (Mogk *et al.*, 2002).

GroEL is a member of the chaperonin family of protein chaperones (Walter, 2002). In order to successfully express and purify Munc 18c from *E. coli* cells it was coexpressed with the chaperonin protein GroEL and protein expression was induced with low levels of IPTG (0.2 mM) at low (22 °C) temperatures overnight as outlined in section 2.4.1 HIS_6-tagged Munc 18c, in pQE30, was expressed in M15 cells that also coexpressed the chaperone GroEL from a separate plasmid. While the GroEL is expressed from a plasmid that contains a kanamycin resistance cassette, pQE30 contains an ampicillin resistance cassette allowing selection of a single colony of *E.coli* expressing both proteins on dual antibiotic plates. HIS_6-Munc 18c was purified using Ni-NTA agarose as outlined in section 2.4.2.

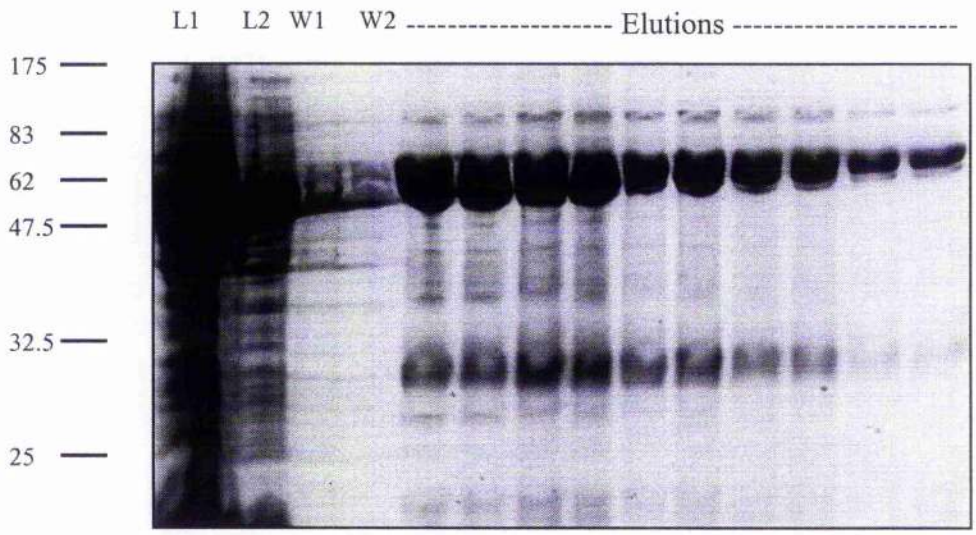


Figure 5.9: Purification of Munc 18c

HIS_6-Munc 18c was expressed from vector pQE30 in M15 expressing GroEL. Expression of HIS_6-Munc 18c was induced overnight at 22 °C by adding 0.2 mM IPTG. The Figure shows the purity of pre-spin lysate (L1), post-spin lysate (L2), wash 1 (W1), wash 2 (W2) and fractions eluted (15 µl) using imidazole (200-500 mM) run on a 12 % SDS-PAGE gel and stained with Coomassie blue as outlined in sections 2.2.1 and 2.2.4. The positions of the broad range molecular weight markers are shown.

As can be seen in Figure 5.9 the elutions from the Ni-NTA agarose contained only one major product of the correct size for HIS_6-full-length Munc 18c, the small band observed below Munc 18c is residually bound GroEL.

Pull downs of the cytoplasmic domains of syntaxin 4 using HIS_6-Munc 18c were unsuccessful as a high degree of non-specific binding between the cytoplasmic domains of syntaxin 4 and the resin was observed (data not shown). Glutathione sepharose bound GST-syntaxin cytosolic domains were therefore employed to pull down HIS_6-Munc18c to overcome this technical difficulty.

5.3.6.2 Purification of GST-syntaxin 4 and GST-syntaxin 4 L173A/E174A

GST tagged syntaxin 4 and syntaxin 4 L173A/E174A were purified as essentially described in section 5.2.3, however, once GST-protein had been bound to glutathione sepharose and unbound protein removed by washing, the protein was not cleaved from the GST tag using thrombin but instead eluted from the beads by incubation with 2 mls of 10 mM reduced glutathione, 50 mM Tris pH8.0 for 30 minutes at 4 °C in the cold room on a rotator. Supernatant containing GST-tagged cytoplasmic domains was collected following a spin at 500 xg for 5 minutes in a bench top centrifuge to pellet the glutathione sepharose beads. GST alone was also expressed from empty pGEX vector and purified as outlined above. The protein was then dialysed overnight against 5 litres of PBS to remove the reduced glutathione. The fusion protein was run on a 12 % SDS-PAGE gel as outlined in section 2.2.1 which was stained with Coomassie blue as described in section 2.2.4 to analyse it for purity. The protein concentration was then estimated as outlined in section 2.4.3.

A gel of the purified proteins used for pull-downs is shown in Figure 5.10.

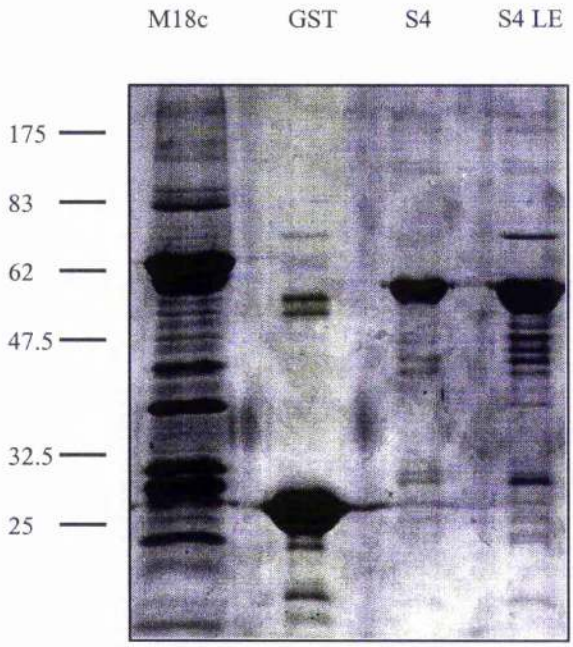


Figure 5.10: Proteins used for GST pull downs

The cytoplasmic domains of syntaxin 4 and syntaxin 4 L173A/E174A along with GST (from empty pGEX vector) were expressed in Rosetta pLysS cells as GST fusions as outlined in section 2.4.1. The proteins were purified by binding the lysate to glutathione sepharose as outlined in section 2.4.2. Following stringent washes the protein was eluted from the glutathione sepharose by incubation with 10 mM reduced glutathione, 50 mM Tris pH 8.0. The protein was dialysed overnight against PBS and run on a 8 % SDS-PAGE gel and stained with Coomassie blue as outlined in sections 2.2.1 and 2.2.4. The positions of the broad range molecular markers are shown. M18c = Munc 18c, GST = GST, S4 = GST-wild type syntaxin 4, S4 LE = GST-syntaxin 4 L173A/E174A.

5.3.6.3 Interaction of Munc 18c with GST-syntaxin 4 and GST syntaxin 4 L173A/E174A

GST-pull down experiments are now a widely established method of investigating interactions between two or more proteins. With the inclusion of appropriate controls they are an ideal method to study the specificity of binding within a protein family. Here, recombinantly expressed proteins were used to establish whether Munc 18c bound to the cytosolic domain of syntaxin 4 and the putatively open mutant of this cytosolic domain, L173A/E174A. Soluble GST was used as the control in these experiments. A set amount of GST or GST-cytosolic domain of syntaxin was immobilised on glutathione sepharose and incubated with increasing amounts of purified HIS_6-Munc 18c. Pull-downs were carried out as outlined in section 2.4.4. Results of a typical experiment are shown in Figure 5.11A. In Figure 5.11B the level of Munc 18c bound to syntaxin 4 and syntaxin 4 L173A/E174A was quantified from the lane where 20 µg of Munc 18c had been incubated with the GST-fusions. The ratio of Munc 18c binding to the GST-syntaxin fusions was calculated. The ratio of Munc 18c bound to syntaxin 4 wildtype was set at 100 % and the ratio of Munc 18c bound to syntaxin 4 L173A/E174A was compared to this.

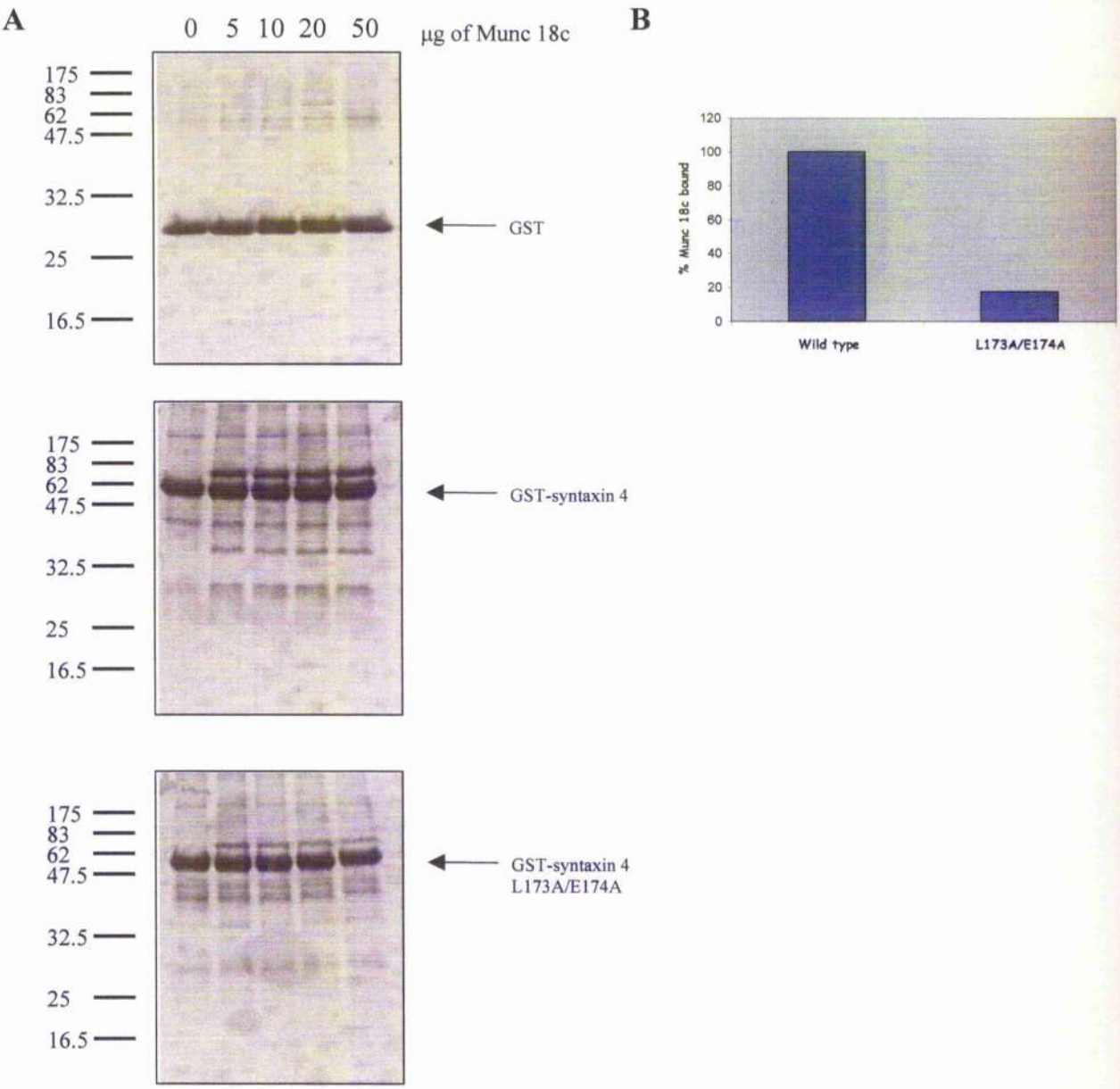


Figure 5.11: Binding of Munc 18c to GST- wild type syntaxin 4 and syntaxin 4 L173A/E174A

Recombinant GST, GST-syntaxin 4 wt or GST-syntaxin 4 L173A/E174A (5 µg) was immobilised onto glutathione sepharose for 1 hour at 4 °C. Unbound protein was removed by washing and the indicated amounts of Munc 18c were incubated with bound protein overnight at 4 °C in 500 µl of binding buffer. Beads were then separated from unbound protein by thorough washing and bound protein was analysed by running on a 12 % SDS-PAGE gel, as outlined in section 2.2.1 which was subsequently stained with Coomassie blue as outlined in section 2.2.4. The positions of the broad range molecular markers are indicated. **(A)** SDS-PAGE of the protein bound to beads **(B)** Quantification of Munc 18c bound to wild type syntaxin 4 and syntaxin 4 L173A/E174A.

As can be seen in Figure 5.11 minimal background binding of Munc 18c was observed with GST alone across all concentrations of Munc 18c added. Munc 18c bound strongly to the cytoplasmic domain of wild type syntaxin 4, at all concentrations tested. However, the introduction of the L173A/E174A mutations into this domain appeared to substantially reduced the binding of Munc 18c, even when Munc 18c was added at a 10:1 ratio to the syntaxin. This agrees with the data that corresponding mutations in syntaxin 1 reduced the binding of Munc 18a to syntaxin 1a (Dulubova *et al.*, 1999). This data suggests that like syntaxin 1a, syntaxin 4 also adopts a closed conformation through which Munc 18c binds suggesting a common mode of binding.

5.4 Discussion

In this chapter I have investigated the conformation adopted by monomeric recombinant syntaxin 4 and the mode through which Munc 18c binds this syntaxin.

SNARE proteins and SM proteins are both indispensable for intracellular membrane fusion. There appears to be three main modes of binding observed between SM proteins and their cognate syntaxins. The first, exemplified by the Munc 18a/syntaxin 1a complex, involves a closed conformation of syntaxin where the SM protein forms an arch shape cradling the syntaxin in this conformation. The second mode involves a short N-terminal peptide of the syntaxin. The third mode involves indirect binding through other proteins.

The fusion of vesicles containing Glut4 to the plasma membrane in insulin responsive cells on stimulation with insulin has been shown to involve the exocytic SNARE complex comprised of syntaxin 4, SNAP23 and VAMP2. The SM protein Munc 18c was cloned from a 3T3-L1 adipocyte cDNA library and has been shown to interact specifically with both syntaxin 2 and syntaxin 4 (Tellam *et al.*, 1997). Like most SM proteins there is conflicting data on the function of this isoform in fusion. While some studies support a negative role in Glut4 exocytosis others have suggested that this SM protein may play a positive role, reviewed in section 1.7.2.4.

Munc 18c has been assumed to bind to syntaxin 4 through a similar mode to that of the binding of Munc 18a to syntaxin 1a, that is it binds to a closed conformation of syntaxin preventing it from entering into SNARE complexes, although this has not been definitively shown. Monomeric syntaxin 1a has been shown by NMR to adopt a default closed conformation (Dulubova *et al.*, 1999). The interaction of Munc 18a appears to hold the protein in this closed conformation (Misura *et al.*, 2000b) preventing SNARE complex formation. Mutations introduced into the hinge region of syntaxin 1a have been shown to flip this syntaxin from the closed conformation to an open conformation which is incapable of binding to Munc 18a (Dulubova *et al.*, 1999). In order to study whether syntaxin 4 adopts a default closed conformation mutations were designed based on the mutations introduced into the cytoplasmic domain of syntaxin 1a and the conformation of syntaxin 4 and mutated syntaxin 4 (L173A/E174A) was studied using CD and limited proteolysis.

Far UV CD showed that the introduction of these mutations into syntaxin 4 had no effect on the overall structural composition of the cytoplasmic domain confirming that the mutations did not cause any gross misfolding of the protein. Near UV analysis of syntaxin 4 and syntaxin 4 L173A/E174A showed that the introduction of these mutations may lead to a less rigid protein conformation. Limited proteolysis, using chymotrypsin, showed that syntaxin 4 L173A/E174A was digested at a more rapid rate than wild type syntaxin 4 suggesting a more accessible, and perhaps open, structure. In order to fully confirm that syntaxin 4 adopts a closed conformation and the L173A/E174A substitutions result in an open conformation a high resolution technique such as NMR or X-ray crystallography could be employed. However this was unfortunately outwith the time restraints of this investigation.

GST-pull downs were used to study the interaction of Munc 18c with the GST tagged versions of wild type syntaxin 4 and syntaxin 4 L173A/E174A. The introduction of these mutations into the hinge region significantly decreases the ability of Munc 18c to bind to syntaxin 4. The corresponding mutations in syntaxin 1a, which have definitively been shown to change the conformation of this protein to open, prevented the binding of Munc 18a to syntaxin 1a (Dulubova *et al.*, 1999). This data suggests that like syntaxin 1a syntaxin 4 also adopts a closed conformation through which Munc 18c binds, suggesting a common mode of binding. There does however, seem to be a low level of Munc 18c binding to syntaxin 4 L173A/E174A above that seen with GST alone. This is not unexpected since low levels of binding of Munc 18a to open syntaxin 1a were also observed using GST pull downs from rat brain lysate (Graham *et al.*, 2004). A weak interaction between the open syntaxin 1a and Munc 18a was also observed using a yeast two-hybrid screen (Dulubova *et al.*, 2003). Using an ELISA technique some binding of Munc 18a to open syntaxin 1a was observed but this was greatly reduced compared to the binding of Munc 18a to wild type syntaxin 1a (Graham *et al.*, 2004). The authors argue that this is due to a difference in maximal binding not affinity. This would agree with the data shown in Figure 5.11, since the level of weak binding of Munc 18c to syntaxin 4 L173A/E174A does not change on increasing the amount of HIS_6-Munc 18c added to the GST pull down.

One explanation as to why a weak interaction is observed between the open syntaxin and Munc 18a is that at any one time a small proportion of the open mutant is in the closed conformation. This has been supported by a recent *in vivo* study using FRET between

Munc 18c and syntaxin 1a which showed that the introduction of the L165A/E166A mutations into syntaxin 1a reduced FRET by approximately 70 % compared to control, suggesting that a proportion of this mutant syntaxin is in a closed conformation (Liu *et al.*, 2004). This may also be the case for syntaxin 4 L173A/E174A and may explain the weak but detectable binding of Munc 18c to this mutant.

So what is the function of this binding of SM proteins to closed syntaxins? The ability of SM proteins to hold exocytic syntaxins in a closed conformation may be a mechanism to regulate the formation of SNARE complexes.

In *C. elegans*, Unc-13 null mutants have impaired evoked synaptic vesicle release (Richmond *et al.*, 2001). This supports a role for UNC-13 in synaptic vesicle priming. The rat homologue of UNC-13, Munc-13 has been shown to interact with the N-terminus of syntaxin and core complexes of syntaxin 1, SNAP25 and VAMP2 (Betz *et al.*, 1997). UNC-13 has been shown to displace the SM protein UNC-18 from *Ce* syntaxin *in vitro* (Sassa *et al.*, 1999). These data suggest that UNC-13 promotes synaptic vesicle priming by either displacing the SM protein UNC-18 or by stabilising the open form of *Ce* syntaxin. The expression of a constitutively open form of syntaxin in *C. elegans* bypasses the requirement for UNC-13 in vesicle priming, while overexpression of wild type syntaxin was without effect in Unc-13 null mutants (Richmond *et al.*, 2001). These data suggest that UNC-13 promotes priming by promoting the opening of syntaxin, perhaps by displacing UNC-18, supporting a negative regulatory role for the SM protein in complex assembly and fusion.

The idea that core complex assembly is regulated by the conformation of syntaxin is supported by the observation that mutations that destabilised the closed conformation of Sso1p significantly enhanced the rate of binary complex formation with Sec9p *in vitro* (Munson *et al.*, 2000) and the formation of ternary complex was also significantly increased *in vivo* (Munson and Hughson, 2002). However, another study showed that once the binary complex is formed the N-terminal domain seems to have no effect on the formation of ternary complex upon addition of Snc2p (Nicholson *et al.*, 1998) suggesting that the closed conformation of syntaxin only serves to regulate the formation of the t-SNARE complex. Mutations which destabilise the closed conformation of syntaxin 1a have not however, been shown to increase the ability of this syntaxin to recruit SNAP25 (Dulubova *et al.*, 1999;Graham *et al.*, 2004). Another possibility is that binding of SM

proteins to a closed conformation of syntaxin may prevent SNARE complexes disassembled by Sec18p/NSF from immediately reassembling.

The elucidation of a common mode of action of SM proteins has been hampered by the lack of overlap between binding studies of members of the SM and SNARE family. Using a yeast two hybrid screen it was shown that the Habc domain of syntaxin 4 was not sufficient to mediate an interaction with Munc 18c and that binding to Munc 18c required the full-length of syntaxin 4 (Grusovin *et al.*, 2000). This suggests that like syntaxin 1a syntaxin 4 exists and interacts with Munc 18c in a closed conformation. However, this Habc domain was missing the N-terminal residues which may mediate binding to Munc 18c (Grusovin *et al.*, 2000). It was also found in this study that the minimal domain of Munc 18c required for binding to syntaxin 4 comprised of only the N-terminal 139 amino acids (Grusovin *et al.*, 2000) consistent with the binding observed between Sly1p and Sed5 (Grabowski and Gallwitz, 1997). Munc 18c has also recently been shown to interact with the t-SNARE complex of syntaxin 4 and SNAP23 as well as the ternary complex of syntaxin 4, SNAP23 and VAMP2 (Latham *et al.*, 2006). The interaction of syntaxin 4 with Munc 18 has been shown to be dependent on the N-terminal of syntaxin 4 since its deletion abolishes binding to Munc 18c (Latham *et al.*, 2006).

In this chapter the conformation of monomeric syntaxin 4 and its interaction with its cognate SM protein Munc 18c was studied. Equivalent mutations to those introduced into syntaxin 1a which caused this isoform to flip from a closed to an open form were introduced into the cytoplasmic domain of syntaxin 4. The introduction of these mutations appeared to lead to a more accessible form of syntaxin 4 suggesting that like syntaxin 1a monomeric syntaxin 4 may exist in a closed conformation. The fact that these mutations also reduced the binding of Munc 18c to syntaxin 4, much like the reduction in binding observed between Munc 18a and mutated syntaxin 1a further supported these syntaxin molecules adopting a similar conformation through which they bind their cognate SNARE protiens. The recent discovery that Munc 18c can bind syntaixn 4 through its N terminus (Latham *et al.*, 2006) suggests the possibility that Munc 18c can bind syntaxin 4 through 2 distinct modes which awaits further investigation.

Chapter 6

Discussion

Although it has been over 25 years since the first demonstration that insulin stimulation caused the mobilisation and translocation of an intracellular store of glucose activity to the plasma membrane of insulin responsive cells (Cushman and Wardzala, 1980;Suzuki and Kono, 1980) the molecular mechanism underlying this phenomenon is yet to be fully characterised. In individuals with type 2 diabetes the ability of insulin to elicit this effect is impaired, therefore investigating the molecular mechanism underlying this is of particular importance. Several advances have been made in this field including the identification of an insulin responsive glucose transporter Glut4 (as outlined in section 1.2.1) which is thought to be held in a population of highly specialised vesicles, and the discovery of many members of the insulin-signalling cascade whose activation leads to the translocation of this isoform (reviewed in section 1.4). However, the regulation of Glut4 trafficking in response to insulin is still not fully understood.

The fusion of Glut4 containing vesicles to the plasma membrane of insulin responsive cells has been demonstrated to be dependent on members of the SNARE family of proteins, namely syntaxin 4 and SNAP23 located at the plasma membrane along with VAMP2 located on Glut4-containing vesicles (reviewed in sections 1.6). SNARE proteins are a family of highly conserved proteins that are known to be essential for all intracellular fusion events. Membrane fusion must be tightly orchestrated in order to maintain the integrity of the different cellular organelles and the formation of SNARE complexes and therefore membrane fusion is thought to be tightly regulated within the cell. Whether the fusion facilitated by the SNARE complex of syntaxin 4, SNAP23 and VAMP2 at the plasma membrane of insulin responsive cells is regulated in response to insulin is yet to be established. However, a recent *in vitro* study using isolated Glut4-containing vesicles and plasma membrane reconstituted into liposomes suggests that this fusion event is indeed regulated by insulin since fusion was greatly increased when plasma membrane from insulin stimulated cells was used as compared to basal plasma membrane (Koumanov *et al.*, 2005). Since this potential point of regulation is a possible defect in diabetes and diabetes is on the substantial increase it is highly interesting to discover more about how this process is regulated.

The fusion process facilitated by SNARE proteins has been successfully reconstituted *in vitro* using recombinant SNAREs purified from *E. coli*, with these SNAREs being both necessary and sufficient to facilitate membrane fusion (Weber *et al.*, 1998). This type of assay is particularly useful since it allows the study of the process of membrane fusion

without the contributions of any other cellular components. As the first step towards studying the regulation of fusion facilitated by the exocytic SNAREs involved in Glut4 translocation, an *in vitro* fusion assay reconstituted with these SNAREs produced as recombinant proteins in *E. coli* was established. In Chapter 3 a fusion assay using these reconstituted liposomes reconstituted definitively showed that these 3 SNARE proteins are necessary and sufficient to facilitate fusion. This has been previously presumed but never proven. This assay may now be used to study the fusion event further.

Although *in vitro* SNAREpins that is the complex between cognate SNAREs, one set on the target membrane and one set on the vesicle membrane, are capable of fusing artificial membranes *in vivo* fusion is believed to be regulated by a number of protein and lipid species. Many members of the SNARE family have been localised to lipid raft domains, isolated as detergent resistant membranes (Schnitzer *et al.*, 1995;Lafont *et al.*, 1999;Chamberlain *et al.*, 2001;Gil *et al.*, 2005). Lipid raft domains are discrete areas of the bilayer which are enriched in cholesterol and sphingolipids, the tight packing of which forms a "liquid ordered state" in comparison to the bulk bilayer which is formed by mainly unsaturated glycerophospholipids which form a "liquid disordered state". The t- and v-SNAREs involved in Glut4 vesicle fusion to the plasma membrane have been localised to lipid raft domains, isolated as detergent resistant membranes, in 3T3-L1 adipocytes (Chamberlain and Gould, 2002). Proteins known to regulate SNARE function were found to be excluded from these domains (Chamberlain and Gould, 2002). Whether lipid rafts play a role in regulating SNARE mediated membrane fusion is a matter of debate. Although experiments where cholesterol was depleted from the membrane of neuronal cells using methyl-β-cyclodextrin suggest that cholesterol rich domains are involved in positively regulating exocytosis (Chamberlain *et al.*, 2001;Lang *et al.*, 2001) in this cell type this data must be treated with caution since cholesterol depletion may have other effects in the cell. A recent study in PC12 cells has shown that the targeting of the SNAP23 homologue SNAP25 to detergent resistant membranes is dependent on its level of palmitoylation (Salaun *et al.*, 2005b) and increased association with rafts inhibited exocytosis (Salaun *et al.*, 2005a).

In Chapter 4 the fusion assay established in Chapter 3 was utilised to study the effect of adding lipids known to be enriched in these detergent resistant membranes to the t-SNARE containing liposomes. The results from these experiments cannot be directly compared as the resulting liposomes from inclusion of these lipids vary drastically in size from the

control liposomes. However, these data do suggest that the inclusion of cholesterol and sphingomyelin into these liposomes is most likely inhibitory to fusion which would agree with the observation that targeting SNAP25 to lipid rafts led to an inhibition of fusion. The transmembrane domains of SNARE proteins have been shown to be important for fusion (Grote *et al.*, 2000;McNew *et al.*, 2000b) and it is possible that the lipid environment surrounding such domains would influence fusion. Lipid raft domains have also been shown to be thicker than the rest of the membrane *in vitro* (Gandhavadi et al., 2002), which may effect the conformation of SNAREs localised to these domains and the fusion that they can support.

The appearance of Glut4 in lipid raft domains in the plasma membrane has recently been shown to precede the appearance in the non-raft fraction suggesting that fusion of Glut4-containing vesicles may occur initially in raft domains (Inoue *et al.*, 2006). Members of the exocyst complex which is responsible for docking the Glut4-containing vesicles to the plasma membrane have also been localised to lipid raft domains suggesting these domains may participate in fusion (Inoue *et al.*, 2006). It is possible that the lipid raft targeting of SNARE proteins in insulin responsive cells serves to spatially organise them in the membrane and bring them into close contact with the insulin signalling pathway, members of which have been localised to these domains, which may regulate complex formation by an as yet unknown mechanism (Reviewed in Saltiel and Pessin, 2003).

SNAP25 and its homologue SNAP23 are known to palmitoylated at conserved cysteine residues within a short sequence on the linker domain separating their two SNARE domains. There is evidence that palmitate may be stimulatory for fusion (Pfanner *et al.*, 1990;Haas and Wickner, 1996;Washbourne *et al.*, 2001) and since palmitoylation is a reversible post-translational modification there is the possibility that palmitoylation of these exocytic SNAREs regulates their ability to support fusion. Although non-palmitoylated SNAREs purified from *E. coli* can facilitate fusion *in vitro* the rate is much slower than that seen *in vivo*. It is possible that the acyl chains of palmitate may distort the membrane leading to more efficient fusion. Most *in vitro* studies including the *in vitro* fusion assay, described in Chapter 3, have so far been carried out using protein purified from *E. coli*. While this is a convenient system for expression of recombinant proteins these proteins are not subjected to the normal eukaryotic post-translational modifications within this prokaryotic host. Recently SNAP25 expressed in insect cells by infection with a recombinant baculovirus has been demonstrated to be palmitoylated (Kammer *et al.*,

2003). In order to express the t-SNARE complex of syntaxin 4 and SNAP23 to purify this for *in vitro* studies in insect cells a recombinant baculovirus was constructed. The baculovirus was successfully created and the t-SNARE complex was purified, with SNAP23 being palmitoylated. However, in comparison to the *E. coli* system the yields were lower and due to time and material constrictions it was unfortunately not possible to perform the fusion assays using this complex.

Although like SNAREs it is thought that all intracellular membrane fusion events require the participation of a member of the SM family the exact role of these proteins in membrane fusion has remained a contentious issue due to conflicting data. In insulin responsive cells Munc 18c is known to interact with syntaxin 4 (Tellam *et al.*, 1997), although like most SM proteins there is conflicting data as to the function of this SM protein in Glut4 translocation as outlined in section 1.7.2.4.

The most widely studied interaction between an SM protein and its cognate syntaxin is the interaction between Munc 18a and syntaxin 1a. Exocytic syntaxins have a highly conserved N-terminal domain, which form an autonomously folded 3 helical bundle termed Habc (Fernandez *et al.*, 1998). In syntaxin 1a this Habc domain is thought to fold back onto the SNARE domain holding the protein in the so-called "closed" conformation which is incapable of entering into SNARE complexes (Dulubova *et al.*, 1999). The crystal structure of Munc 18a in complex with syntaxin1a shows that Munc 18a forms an arch shaped conformation through which it cradles syntaxin 1a (Misura *et al.*, 2000b). It has been proposed from this structure that Munc 18a holds syntaxin 1a in this "closed" SNARE complex incompetent state. The introduction of mutations into the linker domain which joins the Habc domain to the SNARE domain of syntaxin 1a have been shown to flip this syntaxin from the "closed" conformation to a more "open" conformation with the Habc domain and the SNARE domain spatially separated (Dulubova *et al.*, 1999). The "open" conformation is accompanied with the loss of the ability to bind Munc 18a.

In order to investigate whether syntaxin 4 assumes a similar "closed" conformation through which it binds Munc 18c, equivalent mutations were introduced into the cytoplasmic domain of syntaxin 4. The conformations of wild type and mutated syntaxin 4 were studied using CD and limited proteolysis. From these experiments it seems that the introduction of these mutations into syntaxin 4 leads to a more accessible and perhaps open form. This transition from "closed" to "open" needs to be confirmed by a higher resolution

technique for example NMR or X-ray crystallography. Next the interaction of Munc 18c with syntaxin 4 and the mutated version was studied using *in vitro* GST pull-downs. The introduction of these equivalent mutations into the linker domain of syntaxin 4 led to a substantial reduction in the ability of this protein to interact with Munc18c in comparison to the wild type protein. This reduction in binding supports syntaxin 4 adopting a "closed" conformation through which it binds to Munc 18c in a similar fashion to the binding seen for syntaxin 1a to Munc 18a (Dulubova *et al.*, 1999).

A recent study of the interaction between Munc 18c and syntaxin 4 has suggested that Munc 18c binds the N-terminal peptide of syntaxin 4 that precedes the Habc domain (Latham *et al.*, 2006). This mode of binding is similar to that seen for the binding of the SM proteins Sly1p and Vps45p to the syntaxins Sed5p and Tlg2p in yeast (Yamaguchi *et al.*, 2002). It is therefore possible that Munc 18c interacts with syntaxin 4 via two independent modes, the first in a "closed" conformation which Munc 18c cradles (much like that seen for syntaxin 1a and Munc 18a) and the second in an "open" conformation with Munc 18c binding to the very N-terminal of syntaxin 4 (much like the binding observed between the SM proteins Sly1p and Vps45p and their cognate syntaxins Sed5p and Tlg2p). This could explain why the binding of Munc 18c to syntaxin 4 was not completely abolished on introduction of mutations into the hinge region.

In order to investigate further the binding of Munc 18c to syntaxin 4 by these two different modes the affinities of the interactions between Munc 18c, wild type syntaxin 4, syntaxin 4 L173A/E174A and an N-terminal deletion mutant of syntaxin 4 could be studied by Surface Plasmon Resonance, which is a much more sensitive technique than *in vitro* pull-downs. The functional effects of the two different binding modes on fusion could also be evaluated by introducing these two mutants of syntaxin 4 into the *in vitro* fusion assay, as described in Chapter 3, in the presence of Munc 18c. These studies have highlighted novel insights into the interaction between Munc 18c and syntaxin 4 which may influence and regulate the fusion facilitated by syntaxin 4, this awaits further investigation.

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