Insulin-responsive glucose transporter traffic

by

Leonard James Foster

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

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Insulin-responsive glucose transporter traffic

A thesis by Leonard James Foster submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of Biochemistry, University of Toronto, 2001.

Abstract

Insulin stimulates glucose transport in skeletal muscle and adipose tissue by enhancing plasma membrane-directed movement of glucose transporter-4 (GLUT4) via exocytic vesicles. After arrival at the plasma membrane GLUT4 molecules can be recycled to participate in future rounds of exocytosis. Steady-state analysis suggests the involvement of multiple intracellular compartments in this process. Using L6 skeletal muscle cells and 3T3-L1 adipocytes, two main aspects of this traffic are explored in this thesis: endosomal transit of GLUT4 after internalization at the plasma membrane and fusion of the exocytic vesicles with the plasma membrane. The morphological characteristics of compartments transited by GLUT4 are presented here and movement of GLUT4 through these organelles is shown to be accelerated by insulin. The role of SNARE proteins and their binding partners in incorporating GLUT4 exocytic vesicles formed from these organelles into the plasma membrane are also presented. By demonstrating a role for SNAP-23 in GLUT4 arrival at the plasma membrane, SNAP-23 along with VAMP-2 and syntaxin 4 engage as the cognate combination of target- and vesicle-membrane SNAREs required for GLUT4 vesicle fusion. Furthermore, biochemical characterization of these three proteins demonstrates that they possess the capacity to bind one another, a requirement for their ability to mediate vesicle membrane fusion, and do so in a regulated fashion. An additional method of regulation is revealed with the demonstration that the VAMP-binding proteins VAP-33 and pantophysin can also control GLUT4 translocation. In conclusion, insulin may act at an inter-endosomal traffic event to enhance plasma membrane-directed GLUT4 traffic that culminates in fusion of the vesicles with the target membrane through the action of SNAP-23, VAMP-2 and syntaxin 4.
**Preface**

The work presented in this document was performed between September 1996 and October 2000 in the laboratory of Dr. Amira Klip, Programme in Cell Biology, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada with the financial support of the Medical Research Council of Canada/Canadian Institutes of Health Research.

The results of this thesis have been presented in four research articles:


Ideas based on these results have been discussed in two review articles:


**Acknowledgements**

I am forever indebted to many, many people who made the completion of my thesis possible, by providing reagents, financial support, emotional support, or a third (or fourth) hand in order to complete an experiment. A few people made direct contributions to the work to be described here by doing all or part of an experiment and their assistance is footnoted by those particular experiments. Of course, I must thank all members of Klip lab for their help and support over the four years that I worked in the lab, especially Allen Volchuk who taught me many of the techniques I needed to get started. Also, thank you so much to Zayna Khayat for being a sounding board for ideas and for filling in gaps in my knowledge. I also thank the following people from both the Klip and Trimble labs for allowing me to collaborate with them over the years: Karen Yaworsky, Zhi Liu, Dailin Li, Brian Yeung, Dawn Lim, Zayna Khayat, Gary Sweeney, Allen Volchuk, Kathryn Ross, Mahmood Mohtashami, and Lynn Weir. Jeanne Lys-Rafferty, Henry Knight and especially Sheryl Mann provided patient administrative assistance time and time again. Many people have helped me with one technique or another at some point but I would like to acknowledge Mike Woodside, Jeff Butler and Wendy Furuya for all their help with microscopy.

While all the help I received with my work was important, even more meaningful was the emotional support from friends. The move to a new city was not easy but was made much less difficult by all the new friends made here. I must thank Crestina and Mark for all the laughter and good times as well as Kimby for the on-going soap opera. I would especially like to express my gratitude to our new friends Chris and Kate, with whom we now spend a lot of our free time. In addition, it is difficult to conceive what my experience in Toronto would have been like were
it not for Zayna, who has been responsible for at least 50% of my social activities over the years and who has remained a great friend.

A group is only as good as its leader and I was fortunate to have been able to work in a very good group. Sergio Grinstein provided many useful ideas for experiments over the course of my work here. In addition, he allowed me to have a somewhat more comfortable lifestyle by allowing me to manage the Programme’s computers.

My supervisory committee for my degree was as good a council as I could have imagined. Reinhart Reithmeier was always very accommodating, always maintained a pro-student attitude and made many very intelligent adjustments to the direction of my research. In any meeting with my committee I always knew that both he and Bill Trimble could be counted on to ask clear, concise questions and not to try to make me look foolish. In addition to being a terrific committee member, Bill Trimble also provided a good forum for discussing my ideas on the vesicle fusion field and was second only to Amira as the person who I had the most collaborations with.

I know that I cannot express in words how much I appreciate everything that my direct supervisor, Amira Klip, did for me over the past four and a half years. The optimal qualities in a supervisor differ from trainee to trainee but I believe that Amira’s qualities were exactly what I needed in a Ph.D. supervisor. By providing an excellent learning environment she guided me through a (I think) successful and fruitful degree. From very early on she has been a teacher, mentor, and, most importantly, a friend to me.

Of course I would not be here (in more ways that one) were it not for my family, who have provided lots of emotional and some financial support. Finally, I cannot even imagine how different my experience here would have been were it not for my new bride Carmen. She has
supported me through good times and bad times and I am grateful to be spending the rest of my life with her.
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<td>αMEM</td>
<td>α Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor Protein</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical PKC</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>Bo/NT E</td>
<td>Botulinum NeuroToxin serotype E</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine MonoPhosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CI-MPR</td>
<td>Cation-Independent Mannose-6-Phosphate Receptor</td>
</tr>
<tr>
<td>CKII</td>
<td>Casein Kinase II</td>
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<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>EthyleneDiamineTetraAcetate</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Autoantigen 1</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene Glycol-bis(β-aminoethyl ether) –N, N, N’, N’- TetraAcetate</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>ERGIC</td>
<td>ER to Golgi Intermediate Compartment</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine DiPhosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GLUT4</td>
<td>GLUcose Transporter 4</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-Transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine TriPhosphate</td>
</tr>
<tr>
<td>IRAP</td>
<td>Insulin-Responsive AminoPeptidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>KGB</td>
<td>potassium (K) Glutamate Buffer</td>
</tr>
<tr>
<td>LAMP</td>
<td>Lysosomal-Associated Membrane Protein</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>LDM</td>
<td>Low Density Microsomes</td>
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<td>NEM</td>
<td>N-EthylMaleimide</td>
</tr>
<tr>
<td>NSF</td>
<td>NEM-Sensitive Factor</td>
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<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
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<td>PDK</td>
<td>3-Phosphoinositide-Dependent protein Kinase</td>
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<tr>
<td>PH domain</td>
<td>Pleckstrin Homology domain</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-Kinase</td>
</tr>
<tr>
<td>PIPs</td>
<td>Phosphatidylinositol Phosphates</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl-Sulfate PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology domain 2</td>
</tr>
<tr>
<td>SLO</td>
<td>Oxygen-sensitive Streptolysin toxin</td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble NSF-Attachment Protein</td>
</tr>
<tr>
<td>SNAP-23</td>
<td>SyNaptosome-Associated Protein of M, 23,000</td>
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<td>SNAP-29</td>
<td>SyNaptosome-Associated Protein of M, 29,000</td>
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<tr>
<td>SNARE</td>
<td>SNAP REceptor</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin Receptor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi Network</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-Associated Membrane Protein</td>
</tr>
<tr>
<td>VAP-33</td>
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Chapter 1 - Plasma membrane retrieval and endosomal traffic

Introduction

As a dynamic cellular compartment the plasma membrane is constantly turning over its protein and lipid components. For the most part these molecules move through the endosomal system and end up back at the cell surface. This recycling process begins with packaging of the cargo protein into vesicles that go on to fuse with early endosomes (235) and from there move to the recycling endosome and back to the plasmalemma. Not all molecules retrieved from the plasma membrane are reused as some can be targeted for degradation via the late endosome/lysosome pathway. In general, compartments of the biosynthetic pathway (i.e. endoplasmic reticulum (ER), ER to Golgi intermediate compartment (ERGIC) and Golgi) are not heavily involved in the recycling pathway. As a fundamental process of eukaryotic cells, movement of macromolecules through these endosomal systems has been studied extensively. This Chapter summarizes the molecular basis of the individual steps involved in endosome traffic, using as examples the movement of many of the proteins first used to define these pathways. In addition, regulation of some of the steps is discussed as it pertains to this thesis.

1 Throughout this document I have tried to avoid using the term ‘endocytosis.’ While the word literally means ‘into the cell’, it is used ambiguously to describe both movement of something into the cell and movement of something through endosomes. Instead of ‘endocytosis’ to describe movement into the cell I have tried to use ‘internalization’ throughout.
Clathrin-mediated internalization

Clathrin- and caveolae-mediated internalization constitute two major pathways available to cells for retrieving cell-surface proteins. Both are named for the proteins that coat the forming vesicles (clathrin and caveolin) but the two proteins are unrelated, in both structure (primary, secondary, tertiary or quaternary) and function. Clathrin forms a stunning lattice network around the forming vesicle (129, 310), likely helping in its formation and in coordinating its release. Less is known about caveolin and caveolae. Caveolae were originally defined by the marker protein caveolin. That definition has broadened to include a very heterologous collection of plasma membrane domains, some of which may be involved in internalizing cell-surface macromolecules and some that may not. Clathrin-mediated internalization will be focused on in this document.

Originally identified in the mid-1970's (256), clathrin is heterohexamer (also called a triskelion) composed of three heavy and three light chains (173, 357). Mammals contain only two isoforms of clathrin light chain (174) (α and β, which are 60% identical and appear to have redundant functions) and one isoform of clathrin heavy chain. This diversity is not sufficient to explain clathrin's participation in the internalization of a very disparate set of macromolecules. Therefore, adaptor (or assembly) proteins (APs) exist that recognize different subsets of proteins by the presence of specific, juxtamembrane, aromatic residue-based sequences in the cytosolic portions of the molecules (51, 246). AP-2 is the adaptor complex involved in clathrin-coated vesicle formation from the plasma membrane (4) so clathrin collects AP-2-bound plasma membrane proteins into a small area and begins to invaginate the membrane.

At this point numerous other proteins also become involved in orchestrating the formation of the coated vesicles: lipid-modifying enzymes such as synaptojanin and endophilin likely modulate biophysical properties of the membrane thereby relieving stresses induced by
membrane bending (31, 278, 296); dynamin GTPases that help to constrict the bud neck and pinch off the vesicle (134); and scaffold proteins such as amphiphysin and intersectin that provide binding sites and coordination for many other proteins (31, 306). The combined action of these and other proteins constitute a general mechanism for clathrin-mediated internalization.

**Figure 1-1 Endosomal traffic**

The iron (Fe)-transferrin (Tf)-transferrin receptor (TIR) complex and lysosomal enzymes (E) bound to cation-independent mannose-6-phosphate receptor (MR) internalize through clathrin-coated pits. After uncoating the vesicles fuse with early endosomes (EE). The acidic environment inside the early endosomes causes transferrin to release iron which can then be transported into the cytoplasm. The transferrin-transferrin receptor complex is then sorted preferentially into the recycling endosome (RE) where it can move back to the cell surface to pick up more iron. The mannose-6-phosphate receptor typically shuttles lysosomal hydrolases from the trans-Golgi network (TGN) to the late endosome (LE) but can also traffic from the plasma membrane to the late endosomes via early endosomes. The acidic lumenal environment of early endosomes is not sufficiently denaturing to disrupt the interaction between the hydrolases and, in the absence of a sorting signal, the enzyme-receptor complex moves through the default, or bulk flow pathway to the late endosomes. In this organelle, where the pH can be as acidic as 5.0, the lysosomal enzymes are dissociated from the mannose-6-phosphate receptor. The enzymes remain in the bulk flow pathway and remain with the late endosomes as they develop into lysosomes (Lys). The mannose-6-phosphate receptor, however, does not enter lysosomes and is recycled back to the TGN to transport more enzymes.

**Uncoating**

After fission from the plasma membrane, clathrin-coated vesicles must shed their clathrin coat before they are capable of fusing with another organelle. This uncoating is achieved by an
ATPase named uncoating protein (43, 254, 295) which hydrolyzes three ATP molecules for every clathrin triskelion released from a coated vesicle.

**General endosome traffic**

After being uncoated, newly internalized vesicles will rapidly fuse with early endosomes, a peripheral compartment with a tubulovesicular morphology (235). Early endosomes are maintained at an acidic pH (5.9-6.0) by ATP-dependent proton pumps (354), a sufficiently harsh environment to disrupt some ligand-receptor interactions. Antibody-antigen interactions are typically strong enough to resist this denaturation, as are certain other interactions such as that between transferrin and its receptor (235). In the case of macromolecules such as glucose transporters the early endosome represents a transit point in the recycling pathway with no known consequence on their function. As a major hub for vesicle traffic many proteins occupy the early endosome, but most of these occupations are transient. Not surprisingly a number of proteins implicated in vesicle traffic are known to be more permanent residents of early endosomes, including early endosome antigen (EEA1) (233), Rab4 (66), Rab5 (45), Rabip4 (62), syntaxin 13 (264) and syntaxin 7 (265) (see Chapter 2 for functional descriptions of these proteins).

The bifurcation of protein traffic at the early endosome stage is the first sorting step for internalized proteins. The late endosome/lysosome path is the default route for soluble molecules in the lumen of the early endosome but active sorting may be required to target membrane proteins along this route (235). Molecules that do follow this route include certain receptors such as the cation-independent mannose-6-phosphate receptor (CI-MPR), and any soluble molecules trapped inside the lumen of the early endosome that are not attached to a receptor, such as low density lipoprotein (LDL) (235). LDL is carried into the early endosome by the LDL receptor where the acidity dissociates the two macromolecules (68). LDL then moves through the late
endosome to the lysosome while its receptor returns to the cell surface via the recycling pathway (see below).

Like early endosomes, the late endosome is also tubulovesicular in structure but nearer to the centre of the cell while the lysosome is a heterogeneous compartment distributed throughout the cell. The function of CI-MPR (also known as insulin-like growth factor II) is to shuttle hydrolases to the lysosome, usually via the trans-Golgi network (TGN) (180) but occasionally from the plasma membrane as well (152). The acidity of the early endosome is not sufficient to disrupt CI-MPR's interaction with the hydrolases and so, lacking a recycling targeting signal, the whole complex moves on to the late endosome (Figure 1-1). The luminal environment of the late endosome, being more acidic than the early endosome, disrupts the interaction between the lysosomal hydrolases and their CI-MPR chaperone (28). At the late endosome certain transmembrane proteins such as CI-MPR are redirected out of the lysosomal pathway into the TGN (Figure 1-1), leaving the remaining transmembrane proteins and luminal macromolecules to move on to the lysosome. For most macromolecules travelling this route the lysosome represents the terminus since they are degraded by various hydrolases into their basic subunits (amino acids, fatty acids, simple carbohydrates) here, if not earlier in the late endosome (215).

Specific protein markers of the late endosomes include Rabs 7 and 9, CI-MPR (also found in the TGN), and lysosomal-associated membrane proteins (LAMPS) that, as their name suggests, also mark the lysosomes.

The only other known route out of the early endosome for transmembrane proteins is via the recycling endosome, a compartment tubular in nature and often found in a juxtanuclear region associated with the microtubule organizing centre (206, 394). This route is typical of that taken by many cell surface receptors including epidermal growth factor receptor, insulin receptor, LDL receptor and transferrin receptor (235). There is some evidence that the recycling
route is the default pathway for the bulk of internalized plasma membrane, including the lipid
components (184, 216). Transferrin receptor is the prototypical recycled protein, beginning its
cycle at the plasma membrane where it binds iron-loaded transferrin (388). Upon entering the
early endosome the iron is released into the lumen while the apo-transferrin/transferrin receptor
complex is targetted specifically to the recycling endosome (67, 272). From here it can move
back to the plasma membrane, release its apo-transferrin and bind another iron-loaded transferrin
for transport back to the early endosome. Despite this cycle the bulk of a cell's transferrin
receptor complement is found in the recycling endosome, the exact percentage depending on cell
type. Like early endosomes, many proteins are found transiently in recycling endosomes as they
traffic through. However, VAMP-3 (218), syntaxin 13 (350), SNAP-23 (49), Rab4 (350), and
Rab11 (350) are found in recycling endosomes on a more permanent basis along with transferrin
receptor.

**Role of PI3K and PIPs in internalization and endosome traffic**

Numerous proteins involved in internalization and endosome traffic bind inositol
phospholipids including dynamin, synaptojanin and EEA1. Do phosphatidylinositol 3-kinases
and their lipid products control clathrin-mediated internalization? Several studies have found that
the PI3K inhibitor wortmannin has little or no effect on internalization rates of various cell
surface proteins (197, 212, 318). While wortmannin does not appear to affect rates of
internalization, the intimate involvement of the phosphatidylinositol phosphatase synaptojanin in
coated vesicle formation suggests a potential role for inositol phospholipids in this process.
Synaptojanin specifically hydrolyzes phosphates from the D5 position of the inositol ring,
preferring PI[4, 5]P₂ and PI[3, 4, 5]P₃ as substrates. While synaptojanin may be essential for
clathrin-coated vesicle formation (307, 319) it is unclear how or if its enzymatic activity is
involved. Synaptojanin could potentially work by altering phosphatidylinositol kinase signalling,
terminating or altering that information. A second potential effect of the 5-phosphatase activity is to alter membrane lipids, changing their capacity to bind other proteins, including those containing pleckstrin homology (PH) domains. One PH-domain-containing protein that could be a target of regulation is dynamin. However, while dynamin's PH domain is capable of binding PIPs, it appears to be highly specific for PI[4, 5]P2 (285).

While its role in clathrin-mediated internalization remains contentious, PI3K is clearly important for movement of macromolecules through the early endosome. Treatment of cells with concentrations of wortmannin up to 100 nM slows movement of the transferrin receptor out of the early endosome into the recycling endosome and abolishes in vitro homotypic fusion of early endosomes² (158, 197, 212, 316, 318). Wortmannin also alters the morphology of early endosomes, making them longer and more tubular (303). One of the likely early endosomal targets of the PIPs generated by PI3K is the Rab5 effector EEA1 (see Rab Effectors in Chapter 2 for a more detailed description of EEA1). EEA1 contains a FYVE domain that binds PI[3]P specifically in vitro (33, 186, 252). EEA1 is thought to be a tethering protein, drawing two membranes close enough for fusion to occur. Thus, a simple mechanism through which wortmannin might inhibit fusion of early endosomes emerges where the inhibited PI3K is unable to generate a binding site for EEA1, preventing EEA1 from bridging the two membranes.

The use of wortmannin has also implicated PI3K in the exit of proteins out of the recycling compartment back to the cell surface (212, 297, 318). Treatment of cells with 100 nM wortmannin significantly impedes exit of transferrin from the recycling endosome. Little is known at a mechanistic level about how PI3K might mediate traffic events at the recycling endosome (13).

² While this system has provided many insights into the action of PI3K, Rab5 and EEA1, it is not clear what physiological purpose might be served by homotypic fusion of early endosomes. There is no evidence to suggest that early endosomes fuse with one another in vivo or what the function of such a fusion might serve.
Regulation of recycling traffic by extracellular agonists

The recycling pathway is generally though to operate in a constitutive fashion. Surprisingly, little is known about possible regulation by extracellular agonists of the individual steps, i.e. cargo clustering, coated pit formation and vesicle fission, fusion with the early endosome, direction and speed of sorting out of the early endosome, fusion with the recycling endosome and subsequent exit from this compartment. In contrast, regulation of fusion with the plasma membrane has been well studied and is described in more detail in Chapter 2. PI3K and its lipids products (see Role of PI3K and PIPs in internalization and endosome traffic above) as well as the cytoskeleton have demonstrated functions in various recycling steps as shown through pharmacological interference with these molecules (158, 197, 212, 241, 297, 303, 318) and thus exert a form of regulation over those traffic events. However, this form of regulation is distinct from that initiated by an extracellular ligand in that it does not result in changing rates of traffic but merely maintains the constitutive rate. One well-studied case where extracellular ligands regulate the internalization of their receptors is in the down-regulation of certain growth factor receptors and G-protein-coupled receptors by internalization (235). In the absence of ligand the receptors remain on the cell surface but after binding the ligand they begin to internalize. However, once the receptors enter the endosomal pathway they transit the various compartments with the same rates as other molecules who follow those pathways constitutively (235). There are very few reported instances where an extracellular ligand has been observed to modulate the traffic of a macromolecule between two internal compartments. In one report a chimera consisting of the cytosolic and transmembrane domains of P-selectin (a leukocyte-specific cell adhesion molecule) fused to extracellularly-localized horse-radish peroxidase chimera was used to study intercompartmental traffic in pheochromacytoma (PC12) cells (327). In this study the authors report secretagogue (carbamylcholine)-stimulated traffic of the chimera
from dense core granules to the plasma membrane. After arrival at the plasma membrane, the chimera was internalized and arrived in a transferrin receptor-positive compartment from where it underwent secretagogue-dependent traffic into synaptic-like microvesicles (327).

**Sorting of specialized compartments**

In the standard secretory pathway, macromolecules such as insulin (in pancreatic β-cells) or microbicidal enzymes (in polymorphonuclear leukocytes) are packaged into secretory granules directly from the Golgi/TGN (328). This process does not involve the endosomal system, except to retrieve membrane back into the cell after exocytosis. However, there are specialized forms of exocytosis in which the exocytic compartment forms from endosomes such as the well-studied system of synaptic vesicle biogenesis. Filled with neurotransmitters in nerve terminals, synaptic vesicles fuse with the synaptic membrane upon nerve terminal depolarization and release their contents into the synaptic cleft. In order to maintain a pool of synaptic vesicles, neurons must constantly recycle the exocytosed membrane and integral proteins to form new vesicles. While synaptic vesicle recycling is certainly a form of endosomal recycling, it is unclear if it utilizes the same pathways as the conventional endosomal recycling described above. Two lines of evidence suggest that synaptic vesicle recycling involves early endosomes as an intermediate step in their biogenesis. Investigators have labelled synaptic endosomes with soluble markers to facilitate observing them by electron microscopy (130) and examined synaptic vesicles for the presence of Rab5 (83). Results from these studies led investigators to include early endosomes in models of the synaptic vesicle cycle. The major argument against the involvement of the early endosome is a kinetic one: early endosome transit times measured in other cell types are not sufficient to account for the rate of synaptic vesicle biogenesis. More recently, evidence has emerged suggesting that there may be two independent pathways for
synaptic vesicle biogenesis, a rapid one independent of standard endosomes and a slower one involving movement through the endosomal system (178).

Although less studied than synaptic vesicles, a second example of biogenesis of an exocytic compartment from endosomes is that of GLUT4 exocytic vesicle formation in fat and muscle cells. In unstimulated cells GLUT4 cycles continuously to and from the plasma membrane but when challenged with insulin there is a net translocation of GLUT4 from an internal pool to the plasma membrane (151) (see Chapter 3 for a more detailed description of GLUT4 and insulin’s affects on it). During its cycle GLUT4 internalizes via a clathrin- and dynamin-dependent mechanism (5, 165, 373) and likely travels through the recycling endosome at some point in its pathway (211, 270). Beyond this very little is known about the compartments involved in GLUT4 exocytic vesicle biogenesis although studies of the insulin-responsive aminopeptidase (IRAP) suggest that insulin may stimulate budding of vesicles from the recycling endosome. In Chinese hamster ovary (CHO) cells, Johnson et al. observed increased movement of an IRAP/transferrin receptor chimera from recycling endosomes to the plasma membrane (154). This result was confirmed in 3T3-L1 adipocytes by the same group (329). These results are significant to an understanding of GLUT4 traffic because GLUT4 and IRAP traffic is very similar in muscle and adipose cells and so it might be assumed to traffic similarly in other cell types. While exocytic vesicles containing GLUT4 undoubtedly bud from the TGN as well, there is no evidence that this process is regulated by insulin (208), suggesting that its function is to deliver newly synthesized GLUT4 into the cyclical GLUT4 traffic pathway.
Chapter 2 – Exocytic traffic

Identification of proteins involved in vesicle docking & fusion

Seminal work from Rothman’s and Schekman’s groups in the late 1980’s identified a pair of soluble proteins that could bind to the fusing membranes and were required for successful fusion of Golgi vesicles with acceptor Golgi stacks (26, 57, 105, 385). These proteins were termed NSF (N-ethylmaleimide Sensitive Factor) and (SNAP) (Soluble NSF Attachment Protein) based on the sensitivity of the former to N-ethylmaleimide and the ability of the latter to bind to NSF.

Figure 2-1: Common domain structure of SNARE proteins

(a) Syntaxins typically contain three coiled-coil domains. The most C-terminal (juxtamembrane) of these domains contains the conserved glutamine (Q) residue and participates in the SNARE complex. Both VAMPs and syntaxins have an extreme C-terminal transmembrane domain that protrudes through the membrane leaving the C-terminus extracellular (lumenal). Similar to syntaxins, VAMPs also contain a juxtamembrane coiled-coil domain that participates in the SNARE complex but VAMP coiled-coil domains typically contain a conserved arginine (R) residue. SNAP-25 and its homologues contain two coiled-coil domains, one at either end of the molecule, and both domains contain conserved glutamine (Q) residues. In addition, these molecules have multiple cysteine residues in the middle of the molecule that can be palmitoylated to enhance SNAP-25’s interaction with membranes. (b) The three SNARE proteins condense into a complex containing four α-helices, two contributed by SNAP-25 and one each by VAMP and syntaxin. The helices align in a parallel fashion, presumably also parallel to the plane of the membranes. The implication of this is that the flexible linker between the two coiled-coil domains of SNAP-25 must loop back around the complex to allow both domains to align in a parallel fashion.
Independent of this work, many other proteins were being cloned from brain tissues. Some of these proteins included VAMP (Vesicle-Associated Membrane Protein 2) (18, 73, 349), syntaxins A and B (20, 145) and SNAP-25 (SyNaptosome-Associated Proteins of 25 kDa) (250). VAMPs and syntaxins are characterized by a very short extracellularly/lumenally directed C-terminus, a single transmembrane domain and a long cytoplasmic N-terminal region encompassing two coiled-coil domains (Figure 2-1). In contrast, SNAP-25 does not have transmembrane domains, but presents two coiled-coil domains flanking a cluster of cysteine residues that are highly susceptible to palmitoylation (Figure 2-1). The three proteins interact with one-another through their coiled-coil domains, and it is now thought that the binding of SNAP-25 to syntaxin is more relevant to its membrane localization than is its palmitoylation (366, 370). The most convincing evidence that these proteins are involved in vesicle fusion comes from studies using the botulinum and tetanus toxin proteases, which potently inhibit neurotransmitter release. These proteases specifically cleave syntaxin, VAMP and SNAP-25 (24, 200, 291, 292), implicating them in the vesicle fusion process that the toxins inhibit. In the early 1990's Rothman and colleagues demonstrated that NSF and SNAP could bind a complex of syntaxin A, VAMP-2 and SNAP-25 and so termed these proteins SNAP receptors (SNAREs) since they were all membrane bound (314). Subsequently they also showed that ATP hydrolysis by NSF was able to force the dissociation of this complex (313). Based on their topological localization in the presynaptic bouton, these proteins were classified as vesicle (or v-) SNAREs (e.g., VAMP-2) and target (or t-) SNAREs (e.g., syntaxin and SNAP-25).

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3 When this study was done in 1993 syntaxin A and B were the only isoforms of syntaxin known. Since then numerous other isoforms have been cloned and syntaxin A and B are now referred to as syntaxin 1a and 1b respectively and will be referred to as such throughout the rest of this document.
**SNARE hypothesis**

From their original work, Rothman and colleagues proposed that the SNARE proteins would form a link between vesicle and target membranes as a step preceding fusion and that fusion would be driven by the energy released from ATP hydrolyzed by bound NSF (314). Furthermore, given that individual SNARE isoforms were found to have distinct tissular, cellular and organellar specificity, it was proposed that the SNAREs would dictate vesicle targeting specificity (314). The role of SNAREs in targeting fidelity remained a contentious aspect of the SNARE hypothesis for many years but recent evidence now indicates that it is indeed correct (see *Recent advances in vesicle traffic* below). Part of the reason for the debate on targeting specificity was that syntaxin and SNAP-25 populate incoming vesicles, and VAMP is found in target membranes (377). This has led to the suggestion that cis-complexes of v- and t-SNARE may occur within the same membrane, preventing the individual components from engaging in trans-interactions with the opposite membrane. Thus, one of the actions of NSF and αSNAP was found to be to dissociate the cis-complexes using the energy released by ATP hydrolysis (‘priming’, Figure 2-2) (16, 58). The final fusion step depends on SNARE protein integrity but appears to be independent of ATP hydrolysis, suggesting that NSF is not involved at this stage (16, 58).

A structure-function model of fusion has been proposed, whereby SNAREs in the docked conformation ‘zip up’ (‘complex formation’, Figure 2-2), to form a tight, stable SNARE complex (117). The complex involves a four helix coiled-coil bundle now described at atomic resolution (332). In the neuronal SNARE complex SNAP-25 donates two of the helices to this complex while syntaxin 1 and VAMP-2 each contribute one helix. The free energy released by the formation of this exceptionally stable helical bundle is now thought to be the source of energy used to fuse the two lipid bilayers (117) although how this energy is transduced to drive fusion
remains unknown. One model suggested that the energy was used in formation of a continuous α-helix between the transmembrane domains and SNARE complex domains of VAMP-2 and syntaxin4, ripping the transmembrane domains out of the membrane and disrupting the lipid bilayers enough to cause fusion (118, 332). This hypothesis has recently been disproved and the experiments leading to this conclusion are discussed below in Recent advances in vesicle fusion. The elucidation of the crystal structure of the SNARE complex led to an alternative classification of SNAREs into Q- or R-SNAREs, based on the presence of either an glutamine (Q) or arginine (R) residue in the center of the SNARE complex (80). With only two exceptions (vt1b and membrin) Q-SNAREs are equivalent to t-SNAREs and R-SNAREs are equivalent to v-SNAREs (150). For this document the v-/t-SNARE terminology will be used since the function of a particular protein is conveyed more intuitively by this nomenclature than by the Q-/T- system.

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4 In some studies done on neuronal systems syntaxin 1a is still referred to simply as syntaxin. In this document I will refer to the specific syntaxin isoform where it is involved in the results of an experiment. In discussion of models of vesicle fusion however I will use "syntaxin" to refer to the general family of syntaxin proteins and how they are all predicted to work in the model since vesicle fusion models predict that the mechanisms will apply generally the same in different systems. For the same reasons the term 'VAMP' will be used to refer to the general family of VAMP proteins while specific isoforms will be named where they are specified in an experiment.
Figure 2-2: Hypothetical steps in vesicle docking and fusion

Preformed, cis-SNARE complexes on the plasmalemmal and vesicular membranes must first be dissociated by the action of the ATPase NSF and its assistant αSNAP (priming). Following this, the vesicle becomes associated with the plasma membrane (tethering) through as yet unknown molecules. Once tethered, the SNAREs can trans-associate causing the vesicle to become more tightly associated with the plasma membrane (docking). Docking then leads to formation of the classical SNARE complex on the way to fusion of the vesicle with the plasma membrane.
Vesicle docking

Synaptic vesicle fusion is the final step in a series of events that have been described as vesicle tethering (a reversible step) and vesicle docking (an irreversible step) (Figure 2-2). Information on these steps has also emerged from yeast molecular genetic studies (40), and from in vitro endosome-endosome fusion studies (53, 223). The full complement of tethering proteins has not yet been identified but the Early Endosome Autoantigen 1 (EEA1) protein may act as the tethering protein engaged in binding recently internalized vesicles to the early endosome (53, 217) (see Rab effectors below and Chapter 1). EEA1 is found in insulin-sensitive cell-types (253), but its participation in GLUT4 vesicle traffic has not been tested.

Once vesicles are brought into close proximity with their target membranes by the tethering process, SNARE proteins on opposite membranes associate and acquire the configuration required for fusion ('docking', Figure 2-2). The SNARE proteins in docked vesicles are thought to be in a high-energy state (117) that can be maintained for long periods of time. Indeed, large numbers of synaptic vesicles can be observed docked at the presynaptic plasma membrane (150). In contrast, GLUT4-vesicles have rarely if at all been found perched at the plasma membrane of unstimulated muscle or fat cells.

Recent advances in vesicle fusion

As one of the fundamental processes that allow eukaryotes to maintain distinct compartments, the study of vesicle traffic has undergone enormous growth in recent years. Some of the most significant advances in this field include; cloning of SNARE proteins (20, 250, 349), the SNARE hypothesis (314), crystallization of the SNARE complex (332) and, most recently, the development of an in vitro assay to measure the ability of different proteins to mediate fusion between synthetic liposomes (384). The assay involves solubilizing fluorescent phospholipids,
normal phospholipids, and VAMP-2 or just normal phospholipids, syntaxin 1a, and SNAP-25 in a mild detergent. Dilution of this solution below the critical micellar concentration of the detergent forces the phospholipids to form into vesicles and since the hydrophobic domains of the SNAREs are more soluble in a lipid bilayer than in water, they also incorporated into the vesicles. This group reconstituted two different fluorescent phospholipids, N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (rhodamine-PE) into the VAMP-2 vesicles at the same time. The reason for this was that the rhodamine-PE can quench NBD-PE fluorescence in a concentration-dependent manner so dilution of the lipids after vesicle fusion results in increased NBD fluorescence. By mixing vesicles containing VAMP-2, rhodamine-PE and NBD-PE with vesicles containing syntaxin 1a and SNAP-25 with no fluorescent lipids, fusion of the two sets of vesicles can be followed by the increase in NBD-PE fluorescence (384). The development of this assay was a large technological advance in the study of vesicle fusion that has now provided many insights into how SNAREs work and will allow investigators to add back components one by one to completely reconstruct a vesicle fusion system. For instance, in one study Rothman and colleagues demonstrated that the topology of SNAREs in the two opposing membranes further dictates their ability to mediate fusion (251). This means that of the various possible combinations of locations for SNAP-25, syntaxin and VAMP on two different membranes, the only formation that can mediate fusion is the one where SNAP-25 and syntaxin are on one membrane and VAMP is on the other (Figure 2-1b).

One of the hallmarks of eukaryotic cells is their ability to maintain a diverse set of membrane compartments, each containing a unique ensemble of proteins. This is achieved through the specific targeting of vesicles to a diverse set of intracellular compartments. Rothman and colleagues have shown that, with one exception, v-SNAREs can only mediate fusion with t-
SNAREs from the compartment that the vesicle was destined to fuse with (termed 'cognate' pairing). For example, the SNAREs on a Golgi-derived vesicle destined for the plasma membrane can only fuse with the SNAREs on the plasma membrane and not those on the vacuole/lysosome. The original liposome fusion assay was developed using the classical neuronal SNARE combination of SNAP-25, VAMP-2 and syntaxin 1a. The recent results demonstrating the importance of SNARE topology among the two membranes and the fusion specificity dictated by SNAREs were done using SNARE homologues in the budding yeast *Saccharomyces cerevisiae*. Sequencing of the entire *S. cerevisiae* genome led to the identification of all the SNARE proteins in that organism which in turn allowed the elegant demonstration of fusion specificity in the liposome fusion assay. With the sequencing of the *Homo sapiens* genome and other mammalian genomes approaching completion it will be important to confirm this specificity in more complex organisms. The routes between all cellular compartments, including those described above, in which individual SNAREs have been implicated are listed in Table 2-I. Based on the homologous structure/homologous function principle, each SNARE likely functions in a vesicle traffic event but this has not been demonstrated in all cases (see question marks in Table 2-I). On the other hand, some proteins (e.g. syntaxin 7) have been implicated in multiple traffic steps.

The observation that SNAREs do specify targeting fidelity in the *in vitro* assay (219) are consistent with an observation made by Scheller and colleagues at approximately the same time (288). In this study, Scales *et al.* (288) demonstrated that only certain syntaxins and VAMPs could mediate exocytosis of norepinephrine from cracked PC12 cells. Together these results provide support for one of the most hotly contested aspects of the original SNARE hypothesis; that v-SNAREs could only mediate fusion in combination with cognate t-SNAREs.
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<td>Late endosome to lysosome, apical plasma membrane</td>
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5 The references listed here refer to the original demonstration of a FUNCTION for the protein in the indicated traffic vector.
6 Proteins existing only as EST clones are not included (e.g. VAMP-5/6)
7 A question mark (?) is included where the function is only inferred from location, not from functional experiments
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</tbody>
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Some aspects of vesicle fusion are still not fully understood. For instance, based on the crystal structure of the SNARE complex the simplest and most elegant model for how energy released from SNARE complex formation is used to drive membrane fusion is the continuous helix hypothesis (118, 332). As discussed in *SNARE hypothesis* above, this model suggests that the energy release during complex formation is used to mechanically couple the juxtaembrane SNARE complex domains of VAMP and syntaxin with the transmembrane domains of those two proteins. By using forms of VAMP-2 and syntaxin 1a with flexible linkers introduced between the transmembrane and SNARE complex domains McNew *et al.* (220) were able to show that formation of a continuous α-helix in these two proteins was not required for fusion.
Rab proteins

Another family of proteins has been implicated in vesicle traffic and has even more diversity than the SNARE family. The Ras-related Rab family of small molecular weight GTPases contains over 40 proteins (280). Numerous approaches have implicated Rab proteins as critical components of vesicle transport including events as diverse as budding (245, 277), transit along cytoskeletal tracks (71, 240), and docking/fusion (217, 228). The number and specific

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8 There are now multitudes of proteins with real or implied functions in regulating vesicle docking and fusion, most of which are not germane to this thesis. The two most important families, the Rabs and the Munc18 proteins, are described here.
subcellular localizations of Rab isoforms has also led some to suggest that Rabs help to dictate vesicle targeting fidelity (261, 289, 330). However, there is little consensus on the specific aspect(s) of vesicle movement that Rabs are involved in. As often is the case when there is poor agreement on a topic, the lack of consensus may mean that Rabs function in most or all of the aspects of vesicle traffic that they have been implicated. In fact, recent models of vesicle traffic illustrate Rabs as traffic directors, coordinating all the aspects of vesicle movement albeit with little detail as to how this is achieved (12, 103, 280).

Rabs are typically targeted to membranes by twin carboxy-terminus geranylgeranyl groups and, similar to other small molecular weight GTPases, cycle between active and inactive states depending on the form of guanine nucleotide bound (Figure 2-3a). When GTP is bound the GTPase is considered to be 'active' and switches to 'inactive' when it hydrolyzes GTP to GDP. In its active state, Rabs are primarily vesicle membrane-bound while in the inactive state they may be cytosolic or membrane-bound. Like other GTPases in the Ras superfamily, Rabs have a very low intrinsic GTPase activity and require a GTPase-activating protein (GAP) to effectively hydrolyze the γ phosphate from GTP (34). The release of inorganic phosphate occurs around the same time as fusion of the vesicle with its target membrane although the precise sequence of events (SNARE-mediated fusion and GTP hydrolysis by Rabs) has not been determined. Rabs are also like other Ras superfamily members in that they have a high affinity for GDP and require the assistance of a guanine nucleotide exchange factor (GEF) to release the GDP (139), freeing its nucleotide binding site to bind another GTP (Figure 2-3a). However, GDP-dissociation inhibitors prevent this release and escort GDP-Rab back to the membrane it originated from to participate in another round of vesicle traffic (Figure 2-3b) (312).
Rab effectors

The continuously expanding list of putative Rab effectors includes: early endosome antigen 1 (EEA1) and rabaptin5 as Rab5 effectors (305, 326), rabphilin3, Noc2 and Rim as Rab3 effectors (181, 302, 382), rabphilin11 as a Rab11 effector (399) and the Exocyst complex as a Sec4 effector in yeast (109). The Exocyst complex (or rsec6/8 complex) is a large (834 kDa in yeast, 743 kDa in mammals) eight protein complex (108, 141, 342) that may function as a vesicle targeting complex (141, 342). It remains to be shown if there is a mammalian homologue of the Sec15 protein in the yeast Exocyst that binds Sec4, a yeast Rab (109). Without this information the Exocyst complex remains only a yeast Rab effector.

Of the Rab effectors identified, EEA1 has received the most attention. EEA1 was originally identified using an antibody isolated from autoimmune serum of human patients with subacute cutaneous systemic lupus erythematosus (233). Due to its two Rab5-binding domains and single lipid-binding FYVE domain EEA1 is a putative tethering protein that helps to bring two Rab5-containing membranes into closer proximity to allow fusion. EEA1 exists as a parallel homodimer and, in addition to bridging two membranes via its protein-protein and protein-lipid-interacting domains, it also helps to recruit NSF and syntaxin 13 required for fusing the bridged membranes (217). EEA1’s very specific localization to early endosomes suggests that if models of Rab5-mediated membrane traffic are generally applicable to other Rab-mediated events then there must be other effectors such as EEA1 for those systems as well.
Figure 2-4 EEA1 may form a bridge, or tether between opposing membranes linked through Rab5

Through its FYVE (Zn²⁺) domain which binds phosphatidylinositol 3-phosphate, and two Rab5 binding domains (RBD) EEA1 may form a tether between two opposing membranes helping to bring them close to one another to allow SNARE-mediated fusion (12).

Munc18 proteins

The Sec1/unc18/Munc18 family is a second set of proteins that regulates membrane traffic. Sec1 was originally identified as a temperature-sensitive mutant in yeast that was deficient in its secretion ability (243, 244) and cloned in 1991 (2). Homologues of Sec1 were found in other species and given different names in each case (98, 122, 123, 136, 140, 166, 259, 286, 341). The ubiquitous members of this protein family were named Munc18b and c (341) so as the study of vesicle traffic moves beyond the synaptic vesicle field these proteins are now known generically as Munc18 with a few exceptions (150). That Munc18 proteins are required for vesicle fusion has been demonstrated by various means (see Jahn (148) for review), including eliminating its function through genetic knockout (367) and temperature sensitivity (244, 345). Unlike Rabs, more is known about how Munc18 proteins effect their regulation of vesicle traffic, mostly as a result of recent structural studies of Munc18a.

The first clue as to how Munc18 proteins function to regulate vesicle traffic came when Aalto et al. found that a high copy number of the SSO1 and SSO2 genes could suppress mutations in the SEC1 gene (1). This generated excitement in the synaptic vesicle field because Sso1 and Sso2 are yeast homologues of syntaxin 1a and 1b. In the same year Hata et al. reported the identification of Munc18a in a ‘synaptic vesicle fusion complex’ (122) and subsequent studies (10, 70, 98, 123, 136, 166, 259, 337, 340, 341, 396) confirmed the interaction between Munc18 proteins and syntaxins. Munc18 binding to syntaxin appears to preclude binding of
syntaxin to its cognate SNAREs (10, 260, 396). An important advance in understanding how Munc18 proteins work has been the recent derivation of high resolution atomic structures of munc18a bound to the amino-terminus of syntaxin 1a (70, 226). These studies reveal that Munc18-binding to syntaxin forces syntaxin into a 'closed' confirmation that is unable to bind to other SNAREs. Release of Munc18 from syntaxin by an as yet unidentified upstream effector molecule frees the third coiled-coil domain of syntaxin (Figure 2-1a) that can then bind VAMPs and SNAP-25-like proteins to mediate fusion (Figure 2-1b).
Chapter 3 Insulin signalling & GLUT4 traffic

Facilitative glucose transporters

Glucose is the primary molecule used for moving energy among cells. Thus, movement of glucose into cells where it can be used to generate ATP and other products is an essential process since glucose cannot cross lipid bilayers. In eukaryotes this entry is achieved primarily by a family of facilitative glucose transporters that allow glucose to pass through a membrane down a concentration gradient (104, 234, 258). The importance of this facilitation is highlighted by the fact that, for most cases, glucose entry into the cell is the rate-limiting step in its subsequent utilization (72, 106).

Fat and muscle are important peripheral tissues regulating blood glucose levels. After it is secreted from pancreatic β-cells in response to rising blood glucose levels, insulin stimulates fat and muscle cells to remove glucose from the blood stream. GLUT1 and GLUT4 are the two glucose transporters responsible for responding to the insulin challenge in fat and muscle tissues. The individual contributions of GLUT1 and GLUT4 to insulin-stimulated glucose uptake in fat and muscle tissues and cell lines varies. For instance, plasma membranes of rat adipocytes have significantly less GLUT1 than GLUT4 (137), L6 rat skeletal muscle cell line expresses equal levels of the two transporters (387) and 3T3-L1 adipocytes express higher levels of GLUT1 than GLUT4 (39). GLUT1 is ubiquitously expressed, and is primarily localized on the plasma membrane of cells, making it the transporter responsible for basal glucose uptake in most tissues (104, 234, 258). GLUT4 expression is restricted to white and brown adipose tissue as well as skeletal and cardiac muscle (with small levels in brain and kidney) (151) and the role of GLUT4 in regulating blood glucose levels is complex. In the resting state, GLUT4 is harboured in an intracellular compartment in fat and muscle cells and it is recruited from here to the plasma
membrane in response to insulin. Prior to the cloning of GLUT4, the movement of glucose transporter activity from an internal compartment to the plasmalemma was first described by two independent groups in 1980 (65, 333). The gene encoding this transporter was subsequently cloned by several groups in 1989 based on low-stringency hybridization of GLUT1 cDNA to muscle or adipocyte cDNA libraries (23, 44, 95, 151, 160). While GLUT4 is the primary glucose transporter inserted into the membrane with insulin, the hormone also recruits some GLUT1 to the plasma membrane from an internal source. It has long been debated whether insulin only increases the number of glucose transporters in the plasma membrane. Our laboratory has recently demonstrated that insulin also increases the glucose transporting capacity of those glucose transporters at the plasma membrane (334). Whether this activation is achieved through regulation of GLUT1 and/or GLUT4 has not been clearly established but the balance of evidence suggests that it is through regulation of GLUT4 (334).

**GLUT4 structure/function**

Hydropathy plots and biochemical techniques indicate that GLUT4 shares the twelve transmembrane-spanning α-helical domain structure typical of other members of the GLUT family (151). The amino- and carboxyl-termini of the molecule are intracellularly facing, as is a large loop between transmembrane domains 6 and 7. An extended, glycosylated extracellular loop is located between transmembrane domains 1 and 2. The transmembrane domains of GLUT4 that form the glucose channel through the membrane have not been directly identified as most of the structure/function studies of GLUT4 have focused on those regions that must give the molecule its uniqueness among glucose transporters, this is its ability to move to the plasma membrane in response to insulin. The domains responsible for forming the glucose channel have been studied in GLUT1 and include transmembrane domains 7 through 11 (41, 104). Signals in the intracellular portions of a plasma membrane protein, by interacting with clathrin adaptor
proteins, target GLUT4 through the recycling endosome system back to the cell surface (see Chapter 2). In addition, since this recycling is constitutive, a protein such as GLUT4 must have additional signals in its intracellular domains to retain it intracellularly. Various motifs have been identified in GLUT4 that likely regulate its internalization and intracellular retention.

Identification of a motif in the cytoplasmic domain of the LDL receptor that seemed to dictate that protein’s targetting (51) greatly increased our understanding of how proteins are targeted for internalization. Chen et al. showed that an intracellular, juxtamembrane aromatic-based sequence in the cytoplasmic domain of LDL receptor was required for LDL receptor coated-pit internalization. Numerous other integral membrane proteins were also shown to contain similar aromatic-based motifs that could mediate binding of these proteins to adaptor proteins of the internalization machinery (see Chapter 1) (246). The FQIQ motif in the amino-terminal tail of GLUT4 resembles the aromatic-based motif identified in these and other studies (51, 142, 155, 195, 246) and so it has been proposed to mediate GLUT4 internalization (263). Indeed, mutation of the important aromatic (phenylalanine) residue in this motif markedly slows the internalization of GLUT4 in CHO cells (11, 99, 263).

The dileucine-based sorting motif is a second sequence of residues that has been shown to regulate a protein’s movement in the cell. In proteins such as the cation-independent mannose 6-phosphate receptor the dileucine sequence is required for lysosomal targetting (155, 156) while in platelet-derived growth factor beta-receptor the motif allows down-regulation of the receptor via internalization and recycling (230). Such a motif also exists in GLUT4 but there is currently no consensus on the precise role of these residues in GLUT4 traffic. Different reports have implicated the dileucine motif in both GLUT4 internalization (64, 99, 397) and intracellular retention (11, 368). All the studies of the targetting motifs in the cytoplasmic domains of GLUT4 have, by necessity, involved introducing a mutant or chimeric glucose transporter back into
either an insulin-sensitive cell line or a more generic cell line that is then made insulin-sensitive. This raises the question of whether the results of the experiments are truly applicable to understanding the natural system. While this cannot be answered directly from current knowledge, there is substantial evidence from many other systems (using similar techniques) that the targetting domains identified in GLUT4 are functioning as reported (51, 246, 365).

**Insulin signalling**

There are numerous signalling pathways downstream of the activated insulin receptor and these have been reviewed extensively elsewhere (74, 210, 335). Only those pathways leading to the stimulation of GLUT4 translocation to the plasma membrane will be discussed here.

![Insulin signalling](image)

**Figure 3-1 Insulin signalling leading to GLUT4 translocation**

Insulin binding to the insulin receptor (IR) causes the receptor to autophosphorylate, providing binding sites for insulin receptor substrates (IRS) who are in turn phosphorylated by the IR. Phosphorylation of IRS provides a binding site for the regulatory domain of PI3K that then activates the PI3K catalytic domain. PI3K generates phosphatidylinositol phosphates (PIPs) that activate PKB and PDK. Activated PDK phosphorylates PKB (also required for its activation) and aPKCs. From PKB and aPKCs as yet undefined signalling pathways lead to translocation of GLUT4 to the plasma membrane via exocytic GLUT4 vesicles (EGV). Underlying cytoskeleton (gray mesh) provides organization to the whole process.

The insulin receptor is a receptor tyrosine kinase that exists as a heterotetramer containing two α and two β subunits (93, 213) (Figure 3-1). The tyrosine kinase domains in the β
subunits *trans*-autophosphorylate in response to insulin binding (192, 348). The tyrosines phosphorylated in this reaction act as docking sites for src homology 2 (SH2) domains of insulin receptor substrates (IRS) that are also tyrosine phosphorylated by the insulin receptor kinase. These new phosphotyrosines provide docking sites for the SH2 domain of the 85 kDa regulatory subunit (p85) of type I phosphatidylinositol 3-kinase (PI3K). Binding of p85 to phosphorylated IRS causes activation of the p85-bound catalytic subunit of PI3K (p110). Activation of the lipid kinase activity of PI3K results in the production of phosphatidylinositol 3-phosphate (PI[3]P), phosphatidylinositol 3, 4-bisphosphate (PI[3, 4]P2), and phosphatidylinositol 3, 4, 5-trisphosphate (PI[3, 4, 5]P3) (96). A requirement for this kinase in insulin-stimulated GLUT4 traffic was established through the use of the fungal metabolite wortmannin (46, 56, 301, 351) and dominant negative mutants of the enzyme (120, 182, 267). In addition, recent evidence suggests that PI3K can also produce phosphatidylinositol 4-phosphate (96). As the role of individual PI3K products are not well defined in this insulin signalling pathway they will be collectively referred to as PIPs henceforth. The functions of these PIPs in propagating the signals from the insulin receptor are discussed below and in the Cytoskeleton section.

The events leading up to the activation of PI3K by insulin are now well defined but the action(s) of the PI3K products remains contentious. Numerous studies have presented evidence that protein kinase B (PKB) also referred to as Akt, mediates signalling leading to GLUT4 translocation (59, 86, 132, 311, 381) while other studies have implicated atypical isozymes of protein kinase C (aPKC) (14, 15, 183, 322) (Figure 3-1). PKB activation is achieved through a combination of PIPs binding to its PH domain and phosphorylation, by 3-phosphoinositide-dependent protein kinases (PDK, also activated by PIPs), of a serine in its catalytic domain and a threonine in its activation loop. In fact, mutation of these two residues together with a lysine in the substrate binding domain results in a dominant-negative form of the protein (381). Atypical
PKCs are also dependent on PDK phosphorylation of a threonine for activation. While there is no consensus on the role of PKB and aPKCs in GLUT4 traffic, there is substantial evidence implicating both of the enzymes. Thus, it seems likely that both probably function in the pathway, either redundantly or by regulating different aspects of GLUT4 traffic.

**Role of the cytoskeleton in GLUT4 externalization**

The actin cytoskeleton has been repeatedly implicated in exocytic events, both as a barrier separating the docked from stored synaptic vesicles (in essence limiting the active zone), as well as a facilitator of granule exocytosis (22, 360). Recent studies reveal that secretory granules acquire a coat of actin prior to exocytosis (361). Actin filaments are dynamic and, in addition to separating active zones and coating granules, they constitute stress fibers and cortical networks. The latter form in response to growth factor stimulation involving the Rho-family protein Rac (276), and in insulin-sensitive muscle cells they present as large submembranous three-dimensional structures (171, 352). Rac is likely activated by PI3K either directly through binding of PI3K to Rac or indirectly through PIPs activating Rac itself and guanine nucleotide exchange factors (GEFs) which in turn activate Rac (116). Our laboratory has recently shown that formation of cortical actin structures is required for GLUT4 exocytosis (171). Specifically, the rapidly forming subcortical actin mesh contained GLUT4-vesicles and insulin signalling molecules (171). Preventing cortical actin structure formation through transient expression of a dominant negative Rac mutant abrogated externalization of GLUT4 (171). In untransfected muscle cells, GLUT4 is inserted into the membrane at sites of membrane ruffles supported by cortical actin structures (347). These ruffles may represent 'hot zones' of insulin action, similar to active zones of neurotransmitter release seen in neurons, where insulin action is organized (Figure 3-1). In adipocytes, a requirement for an organized cytoskeleton in GLUT4 traffic has also been demonstrated (249).
Whereas actin's role in GLUT4 traffic has been known for many years, the participation of intermediate filaments and microtubules in this process has only come to light recently. Using mass spectrometry, Guilherme et al. identified vimentin and α-tubulin on GLUT4 enriched membranes (107). The same group also found that heterologous expression of hTau40, a protein that impairs kinesin motors, delayed insulin-stimulated GLUT4 movement to the plasma membrane (76). In addition, Tavare and colleagues have reported that disruption of microtubules with vinblastine and colchicine inhibits insulin-stimulated glucose transport and GLUT4 translocation by 40% (84). Together these recent reports provide strong evidence that microtubules and intermediate filaments, along with actin, function in insulin-stimulated GLUT4 traffic.

**GLUT4 compartments**

Does insulin recruit GLUT4 from a pool of vesicles dedicated to GLUT4 traffic in insulin-sensitive cells or does it stimulate formation of export vesicles from the recycling endosome that can then travel to the plasma membrane? The nature of the intracellular compartments occupied by GLUT4 has not yet been described satisfactorily. Density gradient separation of subcellular fractions suggests that there are two or possibly three distinct internal compartments that harbour GLUT4 (6, 121, 138, 193). The relative amount of GLUT4 decreases when these pools are isolated from insulin-treated cells. Inoue et al. recently provided functional evidence that at least one of the compartments isolated by these biochemical approaches is capable of binding to the plasma membrane in response to insulin (146). This group reported the first *in vitro* reconstitution of GLUT4 traffic in an assay that measures docking of intracellular GLUT4 vesicles in with isolated plasma membranes (146).

Subcellular fractionation studies rely on a crude method for identifying compartments, that is, immunoblotting separate fractions and comparing the signal from the protein of interest
with those from accepted markers of known compartments. A more elegant approach to identifying GLUT4 compartments involves eliminating a compartment within living cells by oxidative ablation. This technique has been used successfully in 3T3-L1 adipocytes to ablate the transferrin-containing compartment (presumably the recycling endosome). Treatment of the cells in this manner results in the disappearance of virtually all the intracellular transferrin receptor and 40% of the intracellular GLUT4, suggesting that a portion of the transporter may reside in the recycling endosome (202). There is also evidence that some portion of GLUT4 resides in the TGN (17, 75, 209, 269) but it is unclear if this is newly synthesized GLUT4 progressing through the biosynthetic pathway or if it is actively recycling GLUT4. Notwithstanding these observations, perturbation of the TGN does not significantly impede insulin’s ability to stimulate glucose uptake (89, 143, 179). Beyond the information described here there is little known about the character of the internal compartments that GLUT4 occupies.

While GLUT4 compartments may not be well characterized morphologically or biochemically, a great deal of effort has been expended to increase our understanding of the proteins involved in interacting with the plasma membrane. One approach consisted of identifying the other components of the GLUT4 compartment(s). One of the first proteins identified on GLUT4 vesicles was the prototypical v-SNARE VAMP-2. It was originally detected in immuno-isolated GLUT4 compartments from rat fat cells (36). VAMP-3, a closely related homologue of VAMP-2, is also present on GLUT4-containing compartments (374). However, both proteins populate membranes devoid of GLUT4. Another SNARE protein found on GLUT4 vesicles is syntaxin 4 (375) and the role that this protein plays on these vesicles is not known since the majority of syntaxin-4 resides on the plasma membrane. Other proteins identified on GLUT4 compartments include: insulin-responsive amino peptidase (an enzyme whose traffic mirrors GLUT4) (161, 170, 214), lipid kinases such as phosphatidylinositol 4-
kinase (69) and PI3K (126, 379), Rab4 (63, 358), sortilin (198, 232), endosomal recycling proteins (including transferrin receptor, mannose-6-phosphate receptor and secretory carrier membrane proteins) (163, 190, 202, 343), spectrin (60), amine oxidase (231), pantophysin (32), and a small amount of caveolin (involved in clathrin-independent internalization) (290). The role that these proteins might play in GLUT4 traffic is currently unknown. The functions of VAMP-2, syntaxin 4, pantophysin and rab proteins in GLUT4 traffic are discussed more extensively below.

**Fusion of GLUT4 vesicles with target membranes**

The physiological importance of insulin-dependent GLUT4 translocation to the cell surface has led to numerous attempts to characterize the final GLUT4-vesicle fusion step, drawing on lessons learned from neuronal synaptic transmission. Most studies suggest that the intracellular compartments populated by GLUT4 include the recycling endosome and a specialized vesicular compartment (121, 162, 164, 210). GLUT4-containing vesicles incorporate into the plasma membrane in at least three circumstances: in the basal (unstimulated) state; in response to insulin; in response to exercise in muscle and to insulin in fat cells. This diversity begs the question of whether similar or different molecules participate in GLUT4-vesicle fusion with the plasma membrane in each case. Contrary to neuronal and neuroendocrine cells, muscle and fat cells do not express syntaxin 1, but instead express syntaxin 4 (147, 341, 375). In addition, low levels of syntaxin 2 are also detected in 3T3-L1 adipocytes (375) and rat adipocytes (346) while small levels of syntaxin 3 are present in rat adipocytes (346). Another difference between neuronal/neuroendocrine cells and muscle and fat cells pertains to the expression of SNAP-25. This isoform was not found in insulin-sensitive cells, which instead express SNAP-23 (10, 378, 389).
As discussed in Chapter 2, the t-SNAREs SNAP-25 and syntaxin-1 as well as the v-SNARE VAMP-2 play a central role in synaptic vesicle exocytosis (38, 314). Functional perturbation of any one of these three proteins by a variety of means abolishes or significantly decreases neurotransmitter release (111, 144, 191, 225). SNAP-23, VAMP-2 and syntaxin 4 constitute another 'cognate' set of SNAREs. SNAP-23, a SNAP-25 homologue, was originally cloned from human B lymphocytes and has a widespread expression outside of neuronal and neuroendocrine cells (273, 389). Syntaxin-4 has also been found in a variety of tissues including brain, lung, spleen, kidney, liver, skeletal muscle and fat (21, 375). VAMP-2, syntaxin 4 and SNAP-23 are likely to be a cognate set of SNAREs since they exist in the same traffic routes and can bind one another (273, 274).

**VAMP-2**

VAMP-2, the prototypical v-SNARE, is common to several systems where vesicle traffic is regulated. These include neurotransmitter release in neural synapses (73), insulin-stimulated GLUT4 translocation in fat and muscle cells (36, 372) and aquaporin-2 translocation in renal collecting ducts (153). VAMP-2 is expressed in muscle (268, 372) and fat cells (36, 374) and by subcellular fractionation of those cells the protein is found to be distributed in similar proportions in the plasma membrane and intracellular membranes (36, 372, 374).

VAMP-2 is susceptible to cleavage by various Clostridial neurotoxins (149). This susceptibility afforded a specific strategy to probe the function of VAMP-2 (and a closely related isoform called VAMP-3/cellubrevin) in GLUT4 traffic. We and others have demonstrated a requirement for VAMP-2 in insulin-stimulated GLUT4 translocation (47, 50, 86, 114, 203, 207, 248, 271, 336). Tetanus toxin and Botulinum toxins B & D introduced into rodent adipocytes by electroporation, single cell microinjection, chemical permeabilization (using Streptolysin-O
toxin) or natural, toxin-mediated uptake (47, 50, 86, 114, 203, 336) reduced by more than half the insulin-stimulated GLUT4 incorporation into the cell surface. In addition, introduction of antibodies raised against various regions of VAMP-2 as well as peptides representing different segments of VAMP-2, also diminished the insulin-dependent arrival of GLUT4 at the plasma membrane of rodent adipocytes (47, 203, 207, 248).

Recent work on the function of VAMPs in GLUT4 traffic has focused on resolving whether VAMP-2 or VAMP-3/cellubrevin is the primary v-SNARE involved in insulin-stimulated GLUT4 translocation. It has been suggested that VAMP-2 is the v-SNARE important for translocation of GLUT4 from the insulin-sensitive compartment, since the cytosolic domain of VAMP-2 but not of VAMP-3/cellubrevin or VAMP-1, reduced insulin-stimulated GLUT4 translocation by half when microinjected into 3T3-L1 adipocytes (207, 222). In addition, transfection of tetanus toxin light chain into L6 muscle cells in culture resulted in 70% inhibition of insulin-dependent GLUT4 arrival at the cell surface (271). Basal levels of cell surface GLUT4 were minimally affected. Co-transfection of tetanus toxin-insensitive mutants of VAMP-2 -but not VAMP-3-- rescued the inhibition (271). These results indicate that it is VAMP-2 and not VAMP-3 that is involved in insulin-stimulated GLUT4 translocation, and that neither protein participates in GLUT4 sorting to the plasma membrane in the basal state.

**Syntaxin 4**

Syntaxin 4 is expressed in muscle and fat cells where it is largely, but not exclusively, located at the plasma membrane (331, 346, 375). The amino acid sequence of syntaxin 4 shares approximately 65% similarity with syntaxin 1a (21). In fact, GLUT4-vesicles contain syntaxin 4 that cannot be explained by contamination from plasma membranes (375). Unlike syntaxins 1 through 3, syntaxin 4 is not susceptible to cleavage by Botulinum toxin C1 (293). For this reason
studies on the functional role of syntaxin 4 in GLUT4 translocation have required the use of antibodies and peptides to perturb the function of syntaxin 4. Microinjection (203, 248, 340), chemical permeabilization (47, 375) and adenoviral overexpression (248) have been used to introduce antibodies directed against syntaxin 4 or soluble domains of the protein. In all cases, the perturbation of syntaxin 4 resulted in approximately 50% inhibition of insulin-stimulated glucose uptake (375) or GLUT4 translocation (47, 203, 248, 340).

SNAP-23

SNAP-23 shares both sequence (57.8%) and structural homology with SNAP-25 (273). A protein cloned from a cDNA library of 3T3-L1 adipocytes, originally named syndet (378), was found to be the murine form of SNAP-23 (321). By subcellular fractionation of muscle and fat cells, this protein is found almost exclusively in the plasma membrane-enriched fraction (378, 389). Prior to the work described, only Bo/NT E has been useful to probe the function of SNAP-23. Bo/NT E has been shown to cleave SNAP-23 in some species, notably the canine isoform (196). In one report the toxin was able to cleave the murine SNAP-23, concomitantly reducing GLUT4 translocation (86) while in another study the toxin was ineffective towards SNAP-23 (205). Clearly these results do not provide conclusive evidence for the participation of SNAP-23 in insulin-stimulated GLUT4 translocation so other methods were used to perturb SNAP-23’s function (see Chapter 3).

NSF and αSNAP

Unlike the expanded nature of the SNARE protein families, NSF and αSNAP have very few apparent homologues. High-resolution X-ray crystal structures suggest that NSF may engage αSNAP as a lever to pry the SNARE complex apart (398). This would then allow SNAREs to
form complexes between opposing membranes that are competent for fusion (see Priming in Chapter 2). Indeed, NSF and αSNAP are found in rat adipocytes, and epitope-tagged versions of these proteins have been used to immunoprecipitate SNARE complexes from these cells. Such complexes contained syntaxin 4, VAMP-2, VAMP-3 and SNAP-23 (346). Transfection of a dominant negative mutant of NSF into rat adipocytes resulted in plasma membrane levels of GLUT4 that were not significantly different from basal, untransfected levels (133). However, the level of plasma membrane GLUT4 in basal cells expressing the dominant negative NSF was also lowered significantly (133).

**Ancillary proteins participating in GLUT4-vesicle fusion**

There is now mounting evidence that SNAREs alone can catalyze membrane fusion. However, this remarkable event occurs in many different processes in eukaryotes, some regulated and some not, indicating that more proteins may be involved in some systems in order to provide the regulation. Indeed, many candidates for such regulators have been cloned and, for some, their roles in vesicle traffic examined. Those proteins pertinent to a discussion of GLUT4 traffic are described here:

**Munc18c**

This protein, originally cloned from a 3T3-L1 cDNA library using munc18a as a probe, interacts specifically with syntaxins 2 and 4 but not syntaxins 1 or 3 (337, 341). Munc18c inhibits the binding of syntaxin 4 to VAMP-2 (340, 344) and SNAP-23 (10) and insulin causes the dissociation of a munc18c/syntaxin 4 complex through an unknown mechanism (344). A prediction of this observation is that once insulin causes the dissociation, syntaxin 4 would be available to bind SNAP-23 and VAMP-2, leading to fusion of the vesicles with the target membrane. Indeed, full-length munc18c introduced into 3T3-L1 adipocytes by adenoviral
transfection inhibited insulin-stimulated glucose uptake and GLUT4 translocation by approximately 50% (337, 344). However, a peptide representing the domain of munc18c that binds to syntaxin 4, when microinjected into 3T3-L1 adipocytes, inhibited fusion of GFP-GLUT4-containing vesicles with the plasma membrane. The peptide appeared to allow GLUT4 vesicles to dock with the plasma membrane without fusing with it. Given that this peptide displaces munc18c-binding to syntaxin 4, these results may suggest that the displaced, endogenous munc18c catalyzes fusion (345).

**Synip**

The recently cloned synip is a syntaxin 4-interacting protein, identified in a 3T3-L1 cDNA library by a yeast two-hybrid screen (224). Synip binding to syntaxin 4 prevents VAMP-2/syntaxin 4 binding but not SNAP-23/syntaxin 4 binding (224). As for munc18c, the association of synip with syntaxin 4 is reduced in insulin-stimulated cells. Insulin-sensitivity is conferred by the N-terminal half of synip, whereas the C-terminal half modulates GLUT4 translocation (224). Despite having unrelated primary sequences, synip and munc18c regulate the availability of syntaxin 4 for fusion of GLUT4-vesicles with the plasma membrane in response to insulin. It will be interesting to determine if the two proteins regulate different functional pools of syntaxin 4.

**SNAK**

SNAP-23 kinase (SNAK) is a protein kinase identified by its ability to bind syntaxin 4 in a yeast two-hybrid assay (35). However, SNAP-23 is a better substrate of SNAK than syntaxin 4 (35). SNAK phosphorylates SNAP-23 in vivo and in vitro, specifically SNAP-23 that is not bound to syntaxin 4. SNAK phosphorylation of SNAP-23 enhances t-SNARE complex
assembly, that is, binding of SNAP-23 and syntaxin 4 (35). It is unknown if SNAK is present in insulin-sensitive tissues or if SNAK is activated by insulin.

Hrs-2

The growth-factor-induced phosphoprotein Hrs-2 can bind to SNAP-25 and SNAP-23 in vitro (353). In permeabilized PC12 cells, recombinant Hrs-2 inhibits noradrenaline release (19). Hrs-2 is expressed in muscle and fat cells but its tyrosine phosphorylation state is not altered in response to insulin (K. Yaworsky, L. J. Foster, A. Klip, unpublished observations). To date there is no evidence for its participation as a regulator of GLUT4 traffic.

Pantophysin

A ubiquitous homologue of synaptophysin, termed pantophysin, has recently been cloned from several sources (32, 112). The function of neither protein is well understood although synaptophysin putatively regulates lipid dynamics in synaptic vesicles through its ability to bind cholesterol. Pantophysin is a four membrane-spanning domain protein with both its amino- and carboxyl-termini facing the cytoplasm. Where synaptophysin is localized to synaptic vesicles, pantophysin is found on GLUT4-containing vesicles from 3T3-L1 cells and, like synaptophysin, binds VAMP-2 (32). Interestingly, although pantophysin itself was not phosphorylated, a 77 kDa phosphoprotein associates with pantophysin upon treatment of cells with insulin (32). This result suggests a potential regulation of pantophysin by insulin. This role is explored in a later chapter of this thesis.

VAP-33

A 33 kDa VAMP-2-associating protein (named VAP-33) was isolated from an Aplysia californica cDNA library through a yeast two-hybrid approach (309) and antibodies to VAP-33
prevented synaptic vesicle secretion when injected into *Aplysia* neurons (309). A human homologue was identified soon thereafter (386) and an additional mammalian isoform of VAP-33 was cloned from human brain and rat heart (242) and rat brain (317). The protein originally cloned in Trimble’s laboratory from a pancreatic cDNA library and called hVAP-33 (386) was therefore termed VAP-33a, and second isoform was termed VAP-33b (VAP-33b was called ERG30 in one report (317)). The general domain structure of VAP-33 includes a cytosolic coiled-coil domain and a carboxyl-terminal hydrophobic domain that may represent a transmembrane domain. VAP-33b shares approximately 60% sequence identity with VAP-33a at the amino acid level. In addition, a splice variant of VAP-33b lacking both the coiled-coil and transmembrane domains was described and termed VAP33c (242). In general, all three isoforms appear to be ubiquitously expressed. VAP-33a and VAP-33b bind VAMP-2 *in vitro*, via their transmembrane domains (242, 386). Based on its ability to bind VAMP-2, VAP-33 is a potential regulator of GLUT4 traffic. This role is explored in a later chapter of this thesis.

**Rab proteins**

By genetic complementation, Rab proteins have been implicated in vesicular traffic, specifically in the recognition of vesicles by target membranes (see Chapter 2). To date, only Rab4 has been implicated in GLUT4 traffic by virtue of its presence on immunopurified GLUT4 compartments (63, 358). Insulin stimulation causes Rab4 geranylgeranylation, GTP-loading (298) and dissociation from GLUT4-containing endomembranes (61, 102). Introduction of wild-type or mutants of Rab4 or a peptide representing the hypervariable region of Rab4 resulted in inhibition of insulin-stimulated GLUT4 translocation (61, 299, 376). Interestingly, a link between the Rab-mediated vesicle docking and the actin-based cytoskeleton has been established. Rabphilin, a Rab3 effector, interacts with the actin-bundling protein α-actinin (168),
and Rab8 promotes polarized membrane transport through reorganization of actin filaments (257).

**Rationale & hypothesis**

The significance of understanding GLUT4 traffic is highlighted by the fact that GLUT4 incorporation into the cell surface appears to fail in insulin resistance accompanying several forms of diabetes (172, 176, 401, 402). Clearly there are many steps between insulin binding to its receptor and activated GLUT4 incorporation into the plasma membrane (see Background section III). A defect in any one of these steps could result in less efficient glucose uptake in response to insulin. Thus, a better comprehension of how GLUT4 moves within the cell and how this movement is regulated may lead to more rational therapy approaches for some diabetic patients. Prior to this work there was no demonstrated role for insulin regulation of interendosomal traffic of GLUT4 and the compartments that GLUT4 cycles through were poorly understood. In addition, only VAMP-2 and syntaxin 4 had defined roles as mediators of GLUT4 vesicle fusion with the plasma membrane.

Since GLUT4 is recycled from the plasma membrane it likely follows the same general pathways defined for classical recycling proteins such as the transferrin receptor. Therefore, we explored the spatial and temporal coordinates and tested the hypothesis that insulin might regulate these parameters. The L6 rat skeletal muscle cell line stably expressing extracellularly epitope-tagged GLUT4 (GLUT4myc) allowed us to test these hypotheses by labelling GLUT4myc at the cell surface and following its intracellular traffic. Furthermore, we hypothesized that SNAP-23 likely participated in GLUT4 traffic since it bound VAMP-2 and syntaxin 4 (273). This participation was tested using single-cell microinjection and toxin-mediated plasmalemmal permeabilization to introduce molecules predicted to disrupt SNAP-23
function, using insulin-stimulated GLUT4 translocation as an endpoint measurement. Similarly, the presence of VAP-33 and pantophysin in insulin-responsive tissues (32, 386) and their ability to bind VAMPs (32, 309) suggested that they, too, might regulate GLUT4 traffic. This possibility was tested by microinjecting peptide fragments and antibodies as well as by overexpression of VAP-33. However, since neither VAP-33 nor pantophysin is a SNARE, they are not likely to be membrane fusogens but rather may regulate traffic by controlling VAMP-2 availability.

In summary, the overall purpose of the work described in this thesis was to use biochemical and cell biological techniques to better define the proteins and organelles involved in GLUT4 traffic and their possible regulation by insulin.
Chapter 4 – Analysis of GLUT4 endosomal traffic

Summary

As a recycled protein, GLUT4 likely travels through the early endosome to the recycling endosome and possibly on to a more specialized compartment prior to its recruitment to the plasma membrane in response to insulin. Steady-state analysis of GLUT4 localization supports the notion that GLUT4 populates several intracellular compartments and that insulin can draw GLUT4 from more than one of these compartments. The arrival of GLUT4 at the plasma membrane is the culmination of a cascade of events initiated by insulin but prior to this study the regulation of intracellular GLUT4 traffic by insulin was unknown. In this study we used L6 skeletal muscle cells stably transfected with myc-labelled GLUT4 (GLUT4myc) to follow extracellularly-labelled GLUT4myc from its internalization at the plasma membrane through to its movement through the recycling endosome and examined the effects of insulin on this transit. By using accepted marker proteins of the early and recycling endosomes as well as the trans-Golgi network we were able to follow the progression of extracellularly-labelled GLUT4 through the cells using confocal immunofluorescent microscopy. The results demonstrate that insulin causes a PI3K and PKB-dependent acceleration of movement into and out of the recycling endosome.
**Experimental procedures**

**Materials**

The following antibodies were obtained from commercial sources: Rabbit polyclonal (A-14) and mouse monoclonal (9E10) antibodies against the c-myc epitope (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibodies against human transferrin receptor (Zymed, San Francisco, CA), fluorescein isothiocyanate (FITC)-coupled rat anti-CD25 (Cedarlane Laboratories, Inc., Hornby, ON, Canada), FITC-coupled donkey anti-rat. anti-mouse and anti-rabbit, Cy3-coupled donkey anti-mouse and anti-rabbit and HRP-conjugated donkey anti-mouse secondary antibodies (Bio/Can Scientific, Mississauga, ON, Canada). 9E10 was also obtained as a gift of Carmen de Hoog and Dr. Mike Moran. Rabbit polyclonal antibodies against EEA1 were gifts from Drs. Marino Zerial and Heidi McBride (EMBL, Heidelberg, Germany). TO-PRO3, a fluorescent DNA dye emitting in the far-red region of the spectrum, was obtained from Molecular Probes (Eugene, OR). Poly-L-lysine (>300,000 MW), HEPES-modified RPMI (referred to as HPMI) and o-phenylenediamine dihydrochloride (OPD) were obtained from Sigma (Oakville, ON, Canada). Dako Fluorescent Mounting Solution was obtained from Dako Corporation (Mississauga, ON, Canada).

**DNA constructs**

DNA encoding the extracellular domain of CD25 and the transmembrane and cytoplasmic domains of furin (29) was a generous gift from Dr. Juan Bonifacino (National Institutes of Health, Bethesda, MD). A dominant negative mutant of the p85 α subunit of PI3K (Δp85α) was a generous gift from Dr. Julian Downward (Imperial Cancer Research Fund, United Kingdom). Dominant negative PKB (AAA-PKB) was a generous gift of Dr. James Woodgett.
(Ontario Cancer Institute, Toronto, ON, Canada). Expression of eGFP was achieved using the pEGFP construct from Clonetech (Palo Alto, CA). Purified plasmid DNA of all constructs was prepared using the Midi-prep technique from Qiagen (Mississauga, ON, Canada) according to the manufacturer's directions.

**Cells and Tissue Culture**

A subclone of the L6 rat skeletal muscle cell line stably expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc) has been described previously (355, 380). L6-GLUT4myc myoblasts were grown and differentiated according to previously published protocols (175, 355, 380). Briefly, the cells were cultured in alpha modification of Eagle's medium (αMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin). L6-GLUT4myc cells were passaged by washing with PBS, incubating with 0.05% (w/v) trypsin to lift cells off and diluted into αMEM/FBS. Where indicated, L6-GLUT4myc myoblasts were transiently transfected using the liposome-mediated Effectene reagent (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. Briefly, approximately 1 x 10⁶ cells were seeded in a 75 cm² tissue culture flask one day prior to transfection. On the day of transfection, plasmid DNA was condensed using the Enhancer reagent, then packaged into liposomes consisting mainly of Effectene. These DNA-containing liposomes were then added to the cells for between 5 and 6 hours. The transfection mixture was then replaced with growth medium (10% fetal bovine serum (v/v) in α-MEM) and the cells incubated for a further 38 to 42 hours prior to use in the GLUT4myc endocytosis assay (see below). This transfection protocol consistently resulted in a transfection efficiency of between 10% and 20%. Transfected cells were identified by the presence of cotransfected eGFP. In order to increase the chances that cells
taking up marker constructs also took up the other constructs of interest, 0.4 µg of eGFP DNA was transfected per flask along with 1.6 µg DNA/flask of empty vector, AAA-PKB or Δp85α.

**Cell population assay of GLUT4myc endocytosis**

L6-GLUT4myc cells grown in 24-well tissue culture plates were stimulated with 100 nM insulin at 37°C for 30 min. Cells were then rinsed three times with ice-cold phosphate-buffered saline (PBS) and labelled with 9E10 (8 µg/mL) for 1 h, also at 4°C. After washing four times with ice-cold PBS, α-MEM pre-warmed to 37°C was added to the cells and the plates were floated on a 37°C water bath prior to transferring to a 37°C incubator. Where indicated, 100 nM wortmannin was added during the 1 h incubation with 9E10 and maintained in 100 nM wortmannin throughout the internalization period. Where indicated, 100 nM Insulin was added to the pre-warmed medium used for the internalization. At the indicated times cells were placed on ice, washed once with ice-cold PBS and fixed briefly with 3% formaldehyde in PBS at 4°C for 10 min. After quenching excess formaldehyde with 0.1 M glycine in PBS for 10 min and blocking for 15 min with 5% goat serum in PBS, cells were incubated with HRP-conjugated donkey anti-mouse IgG (1:1,000) at 4°C for 1 h. The cells were then washed six times with PBS and incubated with 1 mL/well OPD (0.4 mg/mL) for 20-30 min at room temperature. The reaction was stopped by addition of 0.25 mL of 3 N HCl and the optical absorbance measured at 492 nm. Cell surface levels of GLUT4myc are expressed as a percentage of the amount of cell surface GLUT4myc on control cells at 0 min of internalization.

**Immunofluorescence in rounded-up myoblasts**

Intracellular traffic of GLUT4myc in L6-GLUT4myc myoblasts was followed in cells replated on poly-L-lysine-coated coverslips. The coverslips were incubated with 200 µg/mL.
poly-L-lysine (MW > 300,000) for 20 min at room temperature. Excess poly-lysine was rinsed off with distilled water and the coverslips were air dried. L6-GLUT4myc cells detached from the substratum using nominally Ca²⁺- and Mg²⁺-free PBS. The cells were released using nominally Ca²⁺ and Mg²⁺-free PBS instead of trypsin in order to preserve the GLUT4myc and insulin receptor molecules on the cell surface. Total removal of Ca²⁺ from the extracellular milieu is also inconsequential to GLUT4 translocation (47). Once the cells started to detach from the substratum, PBS was carefully decanted, HPMI was added and the flask was shaken vigorously to dislodge cells. The cell suspension was aliquoted onto poly-lysine-coated coverslips and allowed to settle for 10 min. Insulin (100 nM) was added to the cells for this 10 minute period in all cases except where indicated in Figure 4-2. The coverslips were transferred to 4°C to halt all vesicular traffic, and then incubated for 1 h at 4°C with HPMI containing anti-myc antibodies (1.33 μg/mL of polyclonal A-14 or 4 μg/mL of monoclonal 9E10 were needed). Coverslips were then washed, maintained at 37°C in the absence or presence of 100 nM insulin then placed directly in ice cold 4% formaldehyde in PBS containing Ca²⁺ and Mg²⁺ for 20 min. The cells were then permeabilized in 0.1% Triton X-100 for 30 min and blocked for 20 min with 5% goat serum. Primary antibodies against TfR (1:1,000) or EEA1 (1:250) were added to the cells for 1 h followed by secondary antibodies (1:250) along with the DNA stain TO-PRO3 (2 μM). Coverslips were mounted on glass slides and imaged using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. Images of Cy5 or TO-PRO3 labeling were given a false blue color to aid in visualization. Over 100 cells were observed per condition per experiment, and representative images of each condition are shown. The extent of colocalization between GLUT4myc and EEA1 or TfR signals was quantified using NIH Image (NIH, Bethesda, MD) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA). The yellow signal generated by overlap between the pair of fluoros was separated from the remaining image using the Select
Color Range filter in Adobe Photoshop 3.0. For panels i to n in Figure 4 the green (GFP) channel of the RGB image was replaced with the blue (TIR) channel for purposes of quantitation. The intensity of the yellow signal selected in this way was then expressed as a percentage of the intensity of the entire GLUT4myc signal.

The use of immunofluorescence and light microscopy for measuring colocalization of two proteins has a number of pitfalls. The most significant issue is the resolution limits of light microscopy. With optimal signal intensities, fluors, etc. the maximum resolution of confocal microscopy is in the range of 200 to 300 nm in the xy plane. This means that two proteins can appear colocalized when in reality they are simply on two separate structures that are within 200 nm of each other. A similar situation occurs in the xz plane where two proteins could be on separate structures that are simply stacked upon one another. Quantitation of fluorescent signals is perhaps not as good a measure of colocalization as simply examining the staining patterns of the two probes. If two signals look identical or at least identical in certain regions that is probably a very good indication that they are in fact colocalized since it is hard to imagine that two entirely separate systems parallel each other throughout the cell. Where two signals look totally different resolution does become an issue since the small amounts of colocalization may be due to real colocalization or simply the proximity of two unresolved but nonetheless separate structures. In this case the interpretation of such data must be approached with caution and (more) scepticism.

Some other very minor issues with light microscopy include spillover and energy transfer. Spillover occurs when light intended to excited one fluorophor actually excites a second fluorophore. This becomes less of an issue with the use of lasers as the excitation source because a very narrow excitation range can be chosen to get specific excitation. Energy transfer can occur when the emission spectra of one fluor overlaps with the excitation of another. In order for this to
occur the fluorors have to be very close together since the efficiency of such a process diminishes
with a factor of $r^a$ where $r$ is the distance between the fluorors. In addition, because of the nature of
the technique there will always be a certain level of operator interpretation that, depending on the
magnitude of the effect being examined, may have a large influence on the conclusions reached.
For all these reasons the degree of colocalization measured in the experiments described below
can only be considered a rough estimate of the actual colocalization of the two proteins.

**Results**

**Rate of disappearance of GLUT4myc from the cell surface**

The rate of disappearance of GLUT4myc from the surface of L6 myoblasts was analyzed
as follows: Cells were stimulated with insulin for 30 min to expose GLUT4myc at the cell
surface. The exofacially exposed myc epitope on the transporters was labelled at 4°C with anti-
myc (9E10) monoclonal antibody for 1 h and the labelled GLUT4myc was then allowed to
internalize upon rewarming to 37°C. Insulin and/or wortmannin were added during the
rewarming step to examine their effects on disappearance of GLUT4myc from the cell surface.
The rate of disappearance of labelled GLUT4myc was determined from the amount of 9E10
antibody remaining at the cell surface and available to react with HRP-conjugated goat-anti-
mouse IgG. Labelled GLUT4myc disappeared from the cell surface with a $t_{1/2}$ of 3 min for all
treatments, fitting a one phase exponential decay curve (Figure 4-1, $r^2$ values: control: 0.970;
insulin: 0.928; wortmannin: 0.999, insulin plus wortmannin: 0.999). Tukey’s pairwise analysis of
variance (ANOVA) of all values at each timepoint indicated that only the 30 min insulin and 30
min insulin plus wortmannin points were significantly different from one another ($p < 0.05$).

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* The experiment described in this section was done by Dr. Dailin Li, a post-doctoral fellow in Amira’s lab. I have
  included it in this work with her permission.
While not statistically significant, the apparent separation of the insulin curve from the other three curves likely reflects labelled GLUT4myc that has internalized, moved through the endosomal system and been reinserted into the plasma membrane. The same reinsertion phenomenon was reported for surface-photolabelled GLUT4 in 3T3-L1 adipocytes using subcellular fractionation (287).

Figure 4-1 Insulin does not affect the rate of GLUT4myc retrieval from the cell surface
Disappearance of GLUT4 from the plasma membrane was measured in untreated (□), insulin-stimulated (▲), wortmannin treated (△), and insulin- and wortmannin treated (●) cells using the cell population assay for GLUT4 endocytosis described in Methods. The absorbance value of control cells at t = 0 was defined as 100% and all other values were normalized to it. The value at 30 min insulin is significantly different from the 30 min value for insulin plus wortmannin (p < 0.05, Tukey's ANOVA). No other values are significantly different from any other in the same time group. Data are mean ± SEM of at least three experiments.

Insulin-induced translocation of GLUT4myc in rounded-up L6 myoblasts

Due to their flattened nature, monolayer cultures are not very amenable for effective resolution of intracellular compartments from dorsal and ventral plasma membranes, even when using confocal microscopy. To overcome this limitation, L6-GLUT4myc myoblasts were detached from their substratum, replated on poly-L-lysine coated coverslips and studied before significant flattening re-occurred. Immediately after replating, the rounded-up cells stood approximately 12 to 15 μm in height and flattened down to 4 to 5 μm after about 40 minutes at 37°C. Cells were permeabilized, then GLUT4myc (Figure 4-2a, d) and transferrin receptor (Figure 4-2b, e) were detected by immunofluorescence microscopy. Nuclear DNA (Figure 4-2c,
f, blue) was labelled using the DNA-intercalating dye TO-PRO3. In both the absence (Figure 4-2a-c) and presence (Figure 4-2d-f) of insulin, TfR was present in a tight compartment near the nucleus, consistent with its classical perinuclear localization (394). In unstimulated cells, a portion of GLUT4myc was localized to the same area as TfR (Figure 4-2c) and only a faint signal was present at the cell surface. Upon 10 min insulin stimulation a distinct rim of GLUT4myc could be observed at cell periphery (Figure 4-2f).

**Figure 4-2 Insulin-induced GLUT4myc translocation in rounded-up myoblasts**

Confocal micrographs of rounded-up cells adhered to poly-L-lysine-coated cover slips in the absence (a-c) or presence (d-f) of 100 nM insulin as indicated (con, ins respectively). Shown are representative images of cells permeabilized prior to staining with antibodies against GLUT4myc (a, d) or TfR (b, e) and combined staining (c, f) of GLUT4myc (red), transferrin receptor (green) and DNA (blue). Confocal micrographs of surface-labelled GLUT4myc (red) in cells at 0 min of GLUT4myc internalization (g, h). Green represents EEA1 staining (g) or TfR staining (h) while DNA is counterstained in blue (g, h). Scale bar represents 10 μm. Shown are representative cells from at least three experiments.

To label only the GLUT4myc molecules on the surface of insulin-stimulated, rounded-up myoblasts, anti-myc antibody was added to unpermeabilized cells at 4°C. Subsequent permeabilization of this population allowed for labelling of DNA and markers of intracellular endosomal compartments. The early endosomal marker EEA1 (Figure 4-2g, green) or the recycling endosomal marker TfR (Figure 4-2h, green) were detected between the rim formed by the surface labelled GLUT4myc (red) and the nucleus (blue).
The difference in remaining surface-labelled GLUT4myc in unstimulated and insulin-stimulated cells presented in Figure 4-1 can also be observed directly by immunohisto-chemistry. Surface-exposed GLUT4myc was labelled, the cells warmed to 37°C for 10 or 20 min and then, without permeabilizing the cells, the myc antibody remaining on the surface was labelled with Cy3-conjugated goat anti-mouse. This method of detection confirms the result from Figure 4-1 that levels of GLUT4myc remaining on the cell surface are not affected by the presence of insulin after 10 min but after 20 min there is more GLUT4myc detectable on the cell surface (Figure 4-3).

**Figure 4-3 Insulin causes GLUT4myc to reappear at the plasma membrane**
Confocal micrographs of remaining surface-labelled GLUT4myc after 10 (a, c) and 20 (b, d) min endocytosis in the absence (a, b) or presence (c, d) of 100 nM insulin (con, ins respectively). Scale bar represents 10 μm. Shown are representative cells from at least four experiments.

**GLUT4myc travels through the early endosome to the recycling endosome**
Morphologically, different endosomes are defined by the presence of marker proteins. Early endosome antigen 1 (EEA1) is a putative tethering protein that helps to bring vesicles in close proximity with the early endosome and is found solely on early endosomes (233).

Numerous proteins undergo constitutive recycling between the plasma membrane and the recycling endosome. The transferrin receptor (TfR) is responsible for iron entry into the cell via binding to transferrin and is constitutively recycled back to the cell surface (388). The relative
abundance of TfR on the plasma membrane and in the recycling endosome varies among cell types. Greater than 90% of the TfR resides in the recycling endosome in L6 myoblasts (L. Foster and A. Klip, unpublished observation), making it a suitable marker for this compartment.

To follow the transit of GLUT4myc through the early endosome and recycling endosome, surface GLUT4myc labeled with anti-myc antibodies was allowed to internalize at 37°C for different times up to 20 min. Figure 4-4a shows the localization of labeled GLUT4myc and EEA1 at the onset of rewarming. Two min after initiation of internalization, some GLUT4myc could be detected in a compartment positive for EEA1 staining (Figure 4-4b) and remained in this compartment for at least 5 min (Figure 4-4c). At 10 min, there was no detectable labeled GLUT4myc in the perinuclear, TfR-positive compartment (Figure 4-4d) but by 15 min (Figure 4-4e) GLUT4myc began to collect there and by 20 min (Figure 4-4f) it reached a steady-state where a large portion of the labeled GLUT4myc overlapped with TfR. This distribution remained for up to 30 min (longer times could not be analyzed due to flattening of the myoblasts).

**Figure 4-4 Internalized GLUT4myc travels through the early and recycling endosomes**

Confocal micrographs of surface-labeled GLUT4myc after 0 (a), 2 (b), 5 (c), 10 (d), 15 (e) and 20 (f) min from the beginning of the internalization period. Red staining is GLUT4myc, blue staining is DNA, and green staining is EEA1 (a-c) or TfR (d-f). Areas outlined by white boxes (a-c) are expanded 4x in insets. Filled arrowheads indicate areas of colocalization (yellow) while open arrowheads highlight areas of non-colocalization. Scale bar represents 10 μm. Shown are representative cells from one of three independent experiments.
Insulin accelerates the transit of GLUT4 through the endosomal system

GLUT4myc was allowed to internalize in the continued presence of insulin. The hormone did not appear to affect the rate of appearance of surface-labeled GLUT4myc in the EEA1-positive compartment (Figure 4-5a-d). Approximately 10% of the GLUT4myc was found in the EEA1-positive compartment (Figure 4-5a-d). In unstimulated cells, only 10% of surface-labeled GLUT4myc was detected in the recycling endosome by 10 min (Figure 4-5e) and this colocalization increased to 37% by 20 min (Figure 4-5f). In contrast, in the continued presence of insulin, 30% of labeled GLUT4myc was already present in the TfR-positive compartment at 10 min after initiation of internalization (Figure 4-5g). Moreover, by 20 min, only 10% of the labeled GLUT4myc internalized in the continued presence of insulin was detectable in the TfR-containing endosomes (Figure 4-5h).

![Confocal micrographs showing GLUT4myc traffic](image)

**Figure 4-5 Effect of insulin on inter-endosomal GLUT4myc traffic**

Confocal micrographs of surface-labeled GLUT4myc after 2 (a, c), 5 (b, d), 10 (e, g) and 20 (f, h) min of endocytosis in the absence (a, b, e, f) or presence (c, d, g, h) of 100 nM insulin (con, ins respectively). Red staining is GLUT4myc, blue staining is DNA, green staining is EEA1 (a, b, c, d) or TfR (e, f, g, h). Filled arrowheads indicate areas of colocalization (yellow) between GLUT4myc and EEA1 or TfR while open arrowheads highlight TfR staining not colocalized with GLUT4myc. Scale bar represents 10 μm. Shown are representative cells from one of six independent experiments. Degrees of overlap (± SE) calculated for any given condition are expressed as the percentage of colocalization out of the total GLUT4myc (red) signal and are indicated below each panel.
Figure 4-6 Acceleration of interendosomal traffic by insulin is dependent on PI3K and PKB

Staining patterns of surface-labeled GLUT4myc (red) in relation to whole-cell TfR (green in a-h, blue in i-n) after 20 min endocytosis in the absence (a, b, e, f, i, k, m) or presence (c, d, g, h, j, l, n) of 100 nM insulin, as indicated (con, ins respectively). Untransfected cells were treated with DMSO (a, c, e, g) or 25 µM LY294002 (b, d, f, h) as indicated. Remaining panels represent cells transiently transfected with empty vector (i, j), Δp85α (k, l) or AAA-PKB (m, n). Transfected cells were identified by the presence of cotransfected eGFP shown in panels (green, i-n). Filled arrowheads indicate areas of colocalization (yellow for a-h, purple for i-n) between GLUT4myc and TfR while open arrowheads point out TfR not colocalized with GLUT4myc. Scale bar represents 10 µm. Shown are representative cells from one of three independent experiments. Degrees of overlap (± SE) calculated for any given condition are expressed as the percentage of colocalization out of the total GLUT4myc (red) signal and are indicated below each panel.
Acceleration of GLUT4myc traffic due to insulin is dependent on PI3K and PKB

PI3K activity is required for insulin-dependent translocation of GLUT4 to the plasma membrane (46, 56, 247, 351, 397), but the exact steps regulated by the enzyme are not known. To explore the role of PI3K in GLUT4 endocytic traffic, surface-labeled GLUT4myc was allowed to internalize for 10 (Figure 4-6a, b, c, d) or 20 (Figure 4-6e, f, g, h) min in the absence (Figure 4-6a, b, e, f) or presence (Figure 4-6c, d, g, h) of insulin, without (Figure 4-6a, c, e, g) or with (Figure 4-6b, d, f, h) cellular pretreatment with the PI3K inhibitor LY294002. In the absence of insulin, LY294002 slightly slowed the time course of movement of labeled GLUT4myc through the recycling endosome (compare Figure 4-6a & b, e & f). In contrast, in insulin-stimulated cells LY294002 retarded the accelerated movement of GLUT4myc through this compartment (compare Figure 4-6c & d, g & h). Therefore, the time course of colocalization of labeled GLUT4myc with TfR was similar for control unstimulated cells as for LY294002-pretreated, insulin-stimulated cells.

To further demonstrate that PI3K is involved in insulin-dependent acceleration of inter-endosomal traffic and to test the role of PKB/Akt in this process, dominant-negative DNA constructs of the two enzymes were transiently transfected into L6-GLUT4myc cells. A construct coding for the p85 subunit of PI3K lacking the intervening SH2 domain that binds the catalytic p110 subunit has been shown to override the insulin-stimulated activation of endogenous PI3K (281) and to inhibit insulin-induced arrival of GLUT4myc to the cell surface (182, 381). We have shown that a construct encoding PKB with three point mutations (K179A, T308A and S473A, called AAA-PKB) overrides the insulin-stimulated activation of cotransfected PKBα (381) and PKBβ (R. Somwar and A. Klip, unpublished results) and also inhibits insulin-dependent exocytosis of GLUT4 (381). Transfection of empty pcDNA3 vector
had no effect on either the unstimulated arrival of labeled GLUT4myc to the recycling endosome (Figure 4-6i) or the insulin-stimulated movement of labeled GLUT4myc through the recycling endosome (Figure 4-6j) (images taken 20 min after initiation of internalization). While neither Δp85α nor AAA-PKB affected the arrival of GLUT4myc to the recycling endosome in unstimulated cells (Figure 4-6k & m respectively), expression of either mutant prevented the accelerated transit of GLUT4myc through the recycling endosome in response to insulin (Figure 4-6l and n). These results suggest that both enzymes participate in the insulin-dependent regulation of this aspect of GLUT4 traffic.

**Internalized GLUT4myc does not travel to the TGN**

Since GLUT4myc travels through the recycling endosome (Figure 4-4) and retrograde transport of some proteins takes place from the recycling endosome to the trans-Golgi network (TGN) (101), it was conceivable that internalized, surface-labelled GLUT4myc might transit through the TGN. To address this possibility, L6-GLUT4myc cells were transiently transfected with a construct encoding the extracellular domain of the high affinity interleukin-2 receptor (CD25) and the transmembrane and cytosolic domains of the endopeptidase furin, a TGN-resident protein (29). After allowing surface-labelled GLUT4myc in transfected myoblasts to internalize, the cells were labelled with anti-CD25 antibody and TO-PRO3. Internalized, surface-labelled GLUT4myc did not colocalize appreciably with furin-CD25 staining at either 0 (Figure 4-7a), 10 (Figure 4-7b) or 20 (Figure 4-7c) minutes of endocytosis and insulin did not change this pattern (results not shown). Instead, surface-labelled GLUT4myc appeared in a TfR-positive, but furin-negative perinuclear compartment after internalization.
**Figure 4-7 Internalized GLUT4myc does not enter the TGN**

Confocal micrographs of surface-labelled GLUT4myc (red), whole-cell furin-CD25 (green) and DNA (blue) after 0 (a), 10 (b) and 20 min (c) endocytosis. Open arrowheads indicate furin-CD25 did not colocalize with GLUT4myc. Scale bar represents 10 μm. Shown are representative cells from at least two experiments.

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**Discussion**

Biochemical and morphological approaches have been used to date in an effort to characterize the endomembranes populated by GLUT4. Gradient centrifugation of adipose cells has revealed at least two distinct intracellular compartments containing GLUT4 (121, 193) as have mathematical models built from measurements of steady state distributions of GLUT4 (138, 193). Moreover, the soluble N-ethylmaleimide sensitive factor attachment protein receptor proteins (SNAREs) vesicle associated membrane protein-2, syntaxin 4 and synaptosome-associated protein of 23 kDa are required for about 50% of the insulin-induced GLUT4 translocation in muscle and fat cells (see (88)), suggesting that insulin draws GLUT4 from two pools distinguishable by their complement of fusogens. Consistent with this scenario, oxidative ablation of transferrin-containing compartments by transferrin-coupled HRP obliterates 30-50% of the intracellular GLUT4 of 3T3-L1 adipocytes (202, 221), indicating that the recycling endosome is one of the compartments populated by GLUT4. Until now there had been no information on whether insulin regulates the traffic through intracellular compartments in either muscle or fat cells. Answering this question is key to our understanding of GLUT4 availability for translocation.
In the present study we measured the rate of disappearance of GLUT4myc from the cell surface, and followed the localization of the transporter once internalized in L6 myoblasts. Insulin did not alter the rate of GLUT4myc removal from the cell surface within 10 min of initiation of endocytosis (Figure 4-1). Once internalized, it took 20 min for GLUT4myc to reach the recycling endosome in the absence of insulin. Strikingly, insulin cut this time by half. Moreover, by 20 min, GLUT4myc internalized in the presence of insulin had exited from the recycling endosome. The PI3K inhibitor LY294002 and a dominant negative form of the p85α subunit of PI3K prevented the early arrival and departure of GLUT4myc to and from the recycling endosome stimulated by insulin. These qualitative observations are supported by quantitative analysis of the overlap between the fluorescent signals representing GLUT4myc and TfR. Quantitation also revealed that treatment of cells with LY294002 alone had a slightly retarding effect on the movement of GLUT4myc through the endosomal system. This is consistent with previous reports that wortmannin slows inter-endosomal movement of transferrin receptor in the presence of wortmannin (212, 318).

While finding surface-labeled GLUT4myc in the recycling endosome was expected, the observed effect of insulin on the inter-endosomal traffic of GLUT4 has not been described before. The findings reported here lead us to propose a revised model of intracellular GLUT4 traffic and its regulation by insulin as presented in Figure 4-8. In this model, internalized GLUT4 travels through the early endosome defined by the presence of EEA1, and progresses to the recycling endosome defined by TfR. Insulin accelerates GLUT4 arrival at the recycling endosome. Our results also suggest that insulin accelerates exit of GLUT4 from this compartment since the residence time of labeled GLUT4myc in the TfR-positive endosome was > 10 min in the absence of insulin but < 10 min in the presence of the hormone. The above results suggest that insulin input is required for at least two distinct functions: movement of
GLUT4 into the recycling endosome (Figure 4-8) and budding out of the recycling endosome. We propose that, from the recycling endosome, GLUT4myc would travel to the cell surface, possibly via generation of specialized exocytic vesicles. Our model also proposes that sorting of GLUT4 occurs in the recycling endosome, but does not rule out that a portion of the exocytic vesicle pool may form directly from the early endosome, since there was always a fraction of the internalized GLUT4myc that did not colocalize with TfR at either 10 or 20 min after internalization.

In a study from 5 years ago (202) a lag time was noted between the disappearance from the plasma membrane of 3T3-L1 adipocytes of the insulin-responsive aminopeptidase (IRAP), whose traffic closely parallels that of GLUT4, and its appearance in a low density microsome fraction, previously shown to contain TfR. It is conceivable that the early endosome defined by EEA1 segregates away from the low density microsomal fraction. In this way, the time differential between disappearance of IRAP from the surface and its appearance in low density microsomes could be due to the transit of IRAP through the early endosome, as demonstrated here for GLUT4myc.

There is general agreement that increased GLUT4 insertion into the plasma membrane in response to insulin requires the action of PI3K (115, 381). In addition, there are several studies suggesting that PKB also contributes to insulin action, especially in L6 skeletal muscle cells (115, 381). However, the precise point(s) of action of PI3K and PKB were unknown, since previous studies only used glucose uptake and/or appearance of GLUT4 in the plasma membrane as end-point measurements to study the effects of perturbing PI3K or PKB mutants. The present study raises the hypothesis that insulin input is required at two distinct loci and is consistent with a model whereby PI3K and PKB mediate both inputs. The overall effect of insulin on inter-endosomal GLUT4 traffic would be to expedite movement of GLUT4 through the endosomal
system, culminating in the genesis of the exocytic GLUT4 vesicles which would then be mobilized to the plasma membrane where they would dock and fuse. This action would provide the cell with a means to maintain levels of plasma membrane GLUT4 in the presence of a continued insulin challenge by regulating the production of plasma membrane-destined GLUT4 vesicles.

Figure 4-8 Model of GLUT4 traffic and sites of insulin input
The results of this study suggest the following model: After removal from the plasma membrane, GLUT4 moves to the early endosome (EE) characterized by EEA1. From the early endosome GLUT4 can travel to the juxtanuclear, recycling endosome (RE) marked by TIR or to the specialized vesicles. Transit to the recycling endosome is regulated by a PI3K- and PKB-dependent signal from insulin. Once in the recycling endosome GLUT4 is presumably packaged into specialized vesicles and this step may also be accelerated by insulin in a PI3K- and PKB-dependent manner. We further hypothesize that GLUT4 can be drawn from the specialized vesicle pool to the plasma membrane by insulin at any time. Our results do not rule out some genesis of GLUT4-specialized vesicles out of the early endosome.
Chapter 5 – Interactions of SNAP-23, syntaxin 4 and VAMP-2

Summary

The SNARE proteins SNAP-23, VAMP-2 and syntaxin 4 have been implicated as mediators of GLUT4 vesicle fusion with the plasma membrane (see Chapter 3) but for SNARE proteins to mediate fusion they must be able to form a tripartite complex. The critical role of SNAP-23 (see Chapter 3), VAMP-2 and syntaxin 4 in GLUT4 integration into the plasma membrane makes them likely candidates for regulation by insulin, either directly through post-translational modifications or indirectly through other proteins. Prior to this study it was unknown if SNAP-23, syntaxin 4 and VAMP-2 could form a complex or how stable such a complex might be. In addition, no information was available on how these three proteins might be regulated by phosphorylation, especially in insulin-sensitive systems. In this study we defined the binding characteristics of SNAP-23, VAMP-2 and syntaxin 4, including binary and ternary interactions among them. Furthermore, we explore the susceptibility of these SNARE proteins to phosphorylation by exogenous protein kinases and the impact of those phosphorylations on binary interactions. More pertinent to the possible regulation of these SNAREs by insulin, syntaxin 4, SNAP-23 and VAMP-2 were tested for susceptibility to phosphorylation by PKCζ and PKBα. The emerging role of the cytoskeleton as a possible organizer of insulin-dependent signals and traffic led us to test the ability of SNAP-23, VAMP-2 and syntaxin 4 to associate with detergent-insoluble, cytoskeleton-enriched biochemical fractions and to concentrate in cytoskeletal structures by immunofluorescent microscopy.

Experimental Procedures

Recombinant Fusion proteins

The following fusion proteins were raised in BS72 *Escherichia coli* using the pGEX-2TK vector (for glutathione S-transferase (GST) fusions) or the pQE30 vector (for polyhistidine (6xHis) fusions): GST-syntaxin 1a cytoplasmic domain, GST-syntaxin 2\textsuperscript{11} cytoplasmic domain, GST-syntaxin 3 cytoplasmic domain, GST-syntaxin 4 cytoplasmic domain, GST-SNAP-23, GST-SNAP-25, 6xHis-VAMP-2-cytoplasmic domain, 6xHis-SNAP-25. Bacteria containing plasmids encoding the above constructs were used to inoculate 100 mL of sterile LB broth containing 100 μg/mL ampicillin the night prior to protein isolation. The following morning the 100 mL culture was diluted into 900 mL of sterile LB and allowed to grow for 1 hr at 37°C. IPTG (0.3 mM) was then added for 4 hr to stimulate expression of the recombinant proteins. Broth containing the bacteria was then centrifuged at 1000 g for 15 min and the resulting bacteria pellets resuspended in lysis buffer (PBS containing 0.05% Tween 20 and 2 mM EDTA for GST proteins, PBS containing 0.05% Tween 20 + 2 μM leupeptin + 2 μM pepstatin A + 200 μM PMSF + 2 mM benzamidine for 6xHis proteins). The suspension/lysate was then passed through a French Press three times to break up any remaining bacteria and clarified by centrifugation at 10,000 g for 10 min. Fusion proteins were then purified by batch method, using glutathione-coupled Sepharose (Sigma, for GST fusion proteins) or Ni-NTA agarose (Qiagen, for 6xHis fusion proteins) beads. Beads were washed three times with PBS containing 0.05% Tween 20 (for GST beads) or with His Wash Buffer (0.2 M NaCl; 10 % Glycerol; 20 mM Imidazole) to remove any lipids or proteins attached non-specifically to the beads. Where needed, GST-fusion proteins were eluted from the beads using 10 mM free, reduced glutathione in 10 mM Tris-HCl.

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\textsuperscript{11} Katherine Ross in Bill Trimble’s laboratory contributed the purified syntaxin 2 and 3 to this study.
pH 8.0. Protein content of successive washes were measured and washes containing greater than 0.1 mg/mL protein were pooled. Polyhistidine-tagged proteins were eluted from Ni-NTA beads by successive washes in increasing concentrations of imidazole, from 100 mM to 250 mM. Fractions were first analyzed for purity by resolving the proteins by SDS-PAGE and staining them with Coomassie Blue. Those fractions where the desired protein represented over 80% of the total protein content were pooled for later use.

Syntaxin 1 and syntaxin 4 were cleaved from GST by incubating 3 µg thrombin/mg GST-syntaxin for 150 minutes at 25°C. SNAP-23 was cleaved from GST by incubating 13 µg thrombin/mg GST-SNAP-23 for 24 h at 4°C. Protein concentration of recombinant proteins bound to glutathione-agarose beads was determined by boiling the beads to remove protein for analysis by SDS-PAGE and comparing the Coomassie blue staining of the supernatant protein with that of known amounts of bovine serum albumin.

**In vitro binding assays**

Binding assays were performed following the procedures described previously (260) for neuronal SNAREs. Briefly, recombinant GST-SNARE fusion proteins (termed the ‘immobilized’ SNARE) was bound to glutathione agarose beads and mixed with soluble recombinant SNARE(s) cleaved from GST as described above. The proteins were incubated in 200 µl of binding buffer (5 mM HEPES pH 7.4, 70 mM KCl, 1 mM MgCl2, and 0.25% Triton-X-100) for 2 h at 4°C with end-over-end rotation. The beads were collected by a low speed centrifugation (10 s, ~1000xg) and washed twice with 1 ml binding buffer. Twenty µl of 2x SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 12 The concentrations of these ‘immobilized’ SNAREs are referred to in standard molar format with the understanding that once bound to beads, the overall concentration will be correct but the local concentration at the bead surface will be much higher.
0.01% bromophenol blue) were added and each tube was boiled for 2-3 minutes for separation by SDS-PAGE on 14% polyacrylamide gels (187).

3T3-L1 adipocyte crude membrane preparation

Monolayers of 3T3-L1 adipocytes were washed twice with ice-cold homogenization buffer (255 mM sucrose, 0.5 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin, 1 mM EDTA, 20 mM HEPES, pH 7.4) then scraped into 4 mL of the same buffer and homogenized by 20 passages through a cell cracker. The homogenate was centrifuged at 1,000 g for 3 min to pellet the nuclei and unbroken cells then the supernatant from this was centrifuged at 245,000 g for 90 min to sediment all the membranes. The pellet containing the membranes was then resuspended in homogenization buffer and the protein content measured by the BCA method (BioRad).

SDS-resistant complexes

Rat brain homogenate solubilized in Triton-X-100 was prepared as described (141). The SDS-resistance of endogenous SNARE complexes was assayed using 20 μg of Triton X-100 solubilized rat brain protein or 90 μg of Triton X-100 solubilized 3T3-L1 mouse adipocyte crude membrane protein incubated at 4°C for 2 h. The SDS-resistance of recombinant protein complexes was tested using equimolar amounts of the recombinant forms of VAMP-2, SNAP-25, SNAP-23, syntaxin 1 and syntaxin 4, all cleaved of GST. The proteins were incubated for 2 h at 4°C in Binding buffer. 2x SDS-sample buffer was added and some of the samples were boiled for 3 min prior to resolution of all samples on SDS-PAGE.

Immunoprecipitation

Monolayers of 3T3-L1 adipocytes were washed twice with PBS and scraped into immunoprecipitation buffer (20 mM HEPES, pH 7.4; 100 mM KCl, 2% Triton X-100, 2 mM
EDTA) containing protease inhibitors (1 μM microcysteine, 1 mM PMSF, 2 μM pepstatin A, 5 μM leupeptin, 10 μg/ml aprotinin) and phosphatase inhibitors (1 mM Na₂VO₄, 20 μM NaF, 10 mM NaPPi) and homogenized by 20 strokes of a glass/teflon homogenizer. The lysates were pre-cleared by incubation for 2 h at 4°C with 40 μl of Protein A-Sepharose. Antibodies to SNAP-23, VAMP-2 and syntaxin 4 (see antibody description below, 2 μg IgG each) were pre-coupled to Protein A-Sepharose by incubation for 2 h at 4°C in immunoprecipitation buffer (20 mM HEPES pH 7.4, 100 mM KCl, 2% Triton X-100, 2 mM EDTA). The pre-coupled beads were pelleted, washed twice with immunoprecipitation buffer and added to the supernatant from the pre-clearing step. Lysate and antibody were incubated for 2 h at 4°C with constant rotation, then the Sepharose was pelleted and washed three times with immunoprecipitation buffer. Fifty μl of SDS-sample buffer were added, the samples were analyzed by SDS-PAGE in 14% polyacrylamide gels, proteins were transferred to nitrocellulose and immunoblotted as described below.

**Protein detection and antibody description**

For SDS-resistance assays and *in vitro* binding assays, proteins were separated by SDS-PAGE, transferred to nitrocellulose and stained with 0.05% Ponceau S in 1% acetic acid for 3-4 min. The nitrocellulose membranes were cut horizontally to separate various SNAREs and immunoblotted with the following antibodies: αSN23.C12, a rabbit polyclonal antibody raised against a hemocyanine-linked peptide comprising the C-terminal 12 amino acids of SNAP-23 (389); αVAMP-2, a rabbit polyclonal antibody raised against a GST-fusion protein encoding the cytoplasmic domain of VAMP-2 (375); αSy4, an affinity purified polyclonal antibody against a GST-fusion protein encoding the cytoplasmic domain of syntaxin 4 (375); SP5, a monoclonal antibody against syntaxin 1 (Serotec, Hornby, ON); C171.1, a monoclonal antibody against
SNAP-25 (generous gift from Dr. R. Jahn, Yale University, New Haven, CT). For the majority of immunoblots, goat-α-mouse-HRP (1:5,000) or goat-α-rabbit-HRP (1:7,500) conjugates were used to detect monoclonal or polyclonal primary antibodies, respectively. In order to confirm the accuracy of ECL, 125I-conjugated protein A (1:10,000) was also used to detect polyclonal primary antibodies. ECL was used to detect binding of horse radish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch) while a PhosphorImager screen was used to detect 125I-protein A. Goat anti-rabbit Cy3 and HRP conjugates were purchased from Jackson Immunoresearch.

**Quantitation of immunoblots**

Bands detected by ECL were scanned with a ScanMaker IIHR flatbed scanner (Microtek, Redondo Beach, CA) and quantitated using NIH Image 1.61 software (National Institutes of Health, USA). Care was taken during exposure of the X-ray film to ensure that all readings were in the linear range. Confirming the quantitative validity of the results, the half maximal binding value measured with ECL (0.55 μM) was found to be comparable to the value measured with 125I (0.57 μM) in one of the individual experiments measuring syntaxin 4:SNAP-23 coupling. Titration curves were fitted and apparent half-maximal binding coefficient values were determined using Origin 4.1 (Microcal). Student's t-tests were performed using Excel 5.0 (Microsoft, Redmond, WA).

**In vitro phosphorylation**

Human recombinant CKII, rat brain PKC (a mixture of α (type III), βI, βII (type II), and γ (type I)) and bovine heart catalytic subunit of PKA were obtained from Boehringer Mannheim (Laval, PQ) and used according to the manufacturer's instructions. To measure phosphorylation
by PKA, 20 pmol of the indicated SNAREs were incubated for 5 min at 30°C in 50 μl of 50 mM MES pH 6.9, 10 mM MgCl₂, 0.5 mM EDTA, 150 μM ATP (0.4 μCi/nmol [γ-32P]ATP) and 1 mM dithiothreitol, with or without 4 mU/ml PKA. To measure phosphorylation by CKII 20 pmol of the indicated SNAREs were incubated 5 min at 37°C in 70 μl of 20 mM MES pH 6.9, 130 mM KCl, 50 μM ATP (0.86 μCi/nmol [γ-32P]ATP), 10 mM MgCl₂, and 4.8 mM dithiothreitol, with or without 0.71 mU/ml CKII. To measure phosphorylation by PKC, 20 pmol of the indicated SNAREs were incubated for 5 min at 30°C in 200 μl of 20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 10 μM ATP (6 μCi/nmol [γ-32P]ATP), 0.5 mM dithiothreitol, with or without 0.25 mU/ml PKC, and with or without the activators 500 μM CaCl₂, 100 μg/ml phosphatidylserine, and 20 μg/ml 1,2-dioleoyl-sn-glycerol (Boehringer Mannheim, Laval, PQ). Where indicated, to maximize phosphate incorporation by PKC 8 pmol of SNAP-25 or SNAP-23 were incubated for 30 min at 20°C in 5 μl of 50 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 50 mM imidazole, 0.06% CHAPS, 100 μM CaCl₂, 50 μM EGTA, 1 mM dithiothreitol, 40 μM ATP (50 μCi/nmol [γ-32P]ATP), with or without 4 mU/ml PKC (Calbiochem, La Jolla, CA).

For preparation of large amounts of phosphorylated syntaxin 4, 100 pmol of agarose-coupled syntaxin 4 were incubated under conditions similar to those described above with the following exceptions: no [γ-32P]ATP was used, the kinase and ATP concentrations were doubled, and the reactions were allowed to proceed for 45 min. The agarose beads were then collected and washed twice with binding buffer to remove reaction components and then used in SNARE binding assays as described above.

For SNARE phosphorylation by endogenous PKB_ and PKC_, methods established previously in our laboratory (315) were modified to use SNARE protein substrates. Briefly, antibodies against the two proteins (Santa Cruz) were first coupled to a 1:1 combination of
Protein A and Protein G-conjugated Sepharose beads (Amersham-Phannacia) in a ratio of 2 μg of antibody per 20 μL of beads. The precoupling was done in PKB Lysis Buffer (50 mM HEPES; pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 30 mM sodium pyrophosphate, 10 mM NaF, 1 mM EDTA) and incubated for 3 h at 4°C, after which the beads were washed twice with PBS and once with PKB Lysis Buffer. Serum-starved cells were then treated with or without 100 nM insulin for 10 min and scraped into PKB Lysis Buffer containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM Na₂VO₄, 1 mM dithiothreitol, 1 μM pepstatin A, 1 μM leupeptin, 100 nM okadaic acid). The cell suspensions were then rotated for 15 min at 4°C to allow the detergent to lyse the cells. Following this the lysates were centrifuged to remove particulate matter and equal volumes of stimulated and unstimulated lysates were rotated with antibody-conjugated beads for 3 h at 4°C. The beads were then washed four times with PKB Wash Buffer (25 mM HEPES; pH 7.8, 0.1% bovine serum albumin, 10% glycerol, 1% Triton X-100, 1 M NaCl) and twice with PKB Kinase Buffer (50 mM Tris-HCl; pH 7.5, 10 mM MgCl₂). For the kinase reaction, 50 pmol of syntaxin 4, GST-SNAP-23, GST-VAMP-2 or GST alone were incubated for 30 min at 30°C with an original 20 μL aliquot of antibody plus immunoprecipitated kinase in PKB Kinase Buffer containing 1 mM dithiothreitol, 5 μM adenosine triphosphate and 2 μCi ³²P-⁻-ATP/sample. The reactions were stopped by addition of 30 μL SDS sample buffer and the entire reaction mixtures were resolved by SDS-PAGE and exposed to a PhosphorImager screen.

Tissue culture.

A subclone of the L6 rat skeletal muscle cell line stably expressing GLUT4 with an exofacial myc epitope was generated as described earlier (175, 355, 380). Parental L6 myotubes, L6-GLUT4myc myoblasts and 3T3-L1 adipocytes were grown and differentiated according to
previously published protocols (227, 355, 374, 380). Briefly, L6-GLUT4myc myoblasts were
cultured in alpha modification of Eagle’s medium (αMEM) supplemented with 10% (v/v) fetal
bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic solution (10,000 units/mL penicillin, 10
mg/mL streptomycin). Parental L6 myotubes were induced to differentiate from myoblasts by
using αMEM with only 2% FBS and used 6 days after initiation of differentiation. 3T3-L1
adipocytes were maintained as fibroblasts in Dulbecco’s modification of Eagle’s medium
(DMEM) supplemented with 10% (v/v) calf serum (CS) and 1% (v/v) antibiotic/antimycotic
solution. 3T3-L1 fibroblasts were induced to differentiate into adipocytes by replacing the
DMEM/CS with DMEM supplemented with 10% FBS, 1% (v/v) antibiotic/antimycotic solution
and 0.25 μM dexamethazone, 0.5 mM 1-methyl-3-isobutylxanthine and 10 μg/mL porcine
insulin. After four days the medium was changed to DMEM/FBS with 10 μg/mL insulin for an
additional two days then replaced every two days with DMEM/FBS. Adipocytes were used in
experiments between 11 and 14 days after initiation of differentiation. All cell types were
passaged by washing with PBS, incubating with 0.05% (w/v) trypsin to lift cells off and diluted
into αMEM/FBS (L6-GLUT4myc myoblasts) or DMEM/CS (3T3-L1) for replating.

**In vivo phosphorylation**

To test for in vivo phosphorylation of SNARE proteins, 6 cm dishes of 3T3-L1
adipocytes monolayers were washed 3x with serum- and P1-free DMEM and then incubate with 3
mL of the same media containing 0.5 mCi of carrier-free $[^{32}P]P_{1}$ per dish for 2 h at 37°C. Cells
were then stimulated with or without 100 nM insulin for 10 min at 37°C, then lysed and VAMP-
2, SNAP-23 and syntaxin 4 were immunoprecipitated from the lysates as described above. Extra
precautions were taken to avoid long exposure to the high levels of radioactivity involved.
Radioactive proteins were detected by separating the immune complexes by SDS-PAGE and
exposing the resulting gels to a PhosphorImager. PhosphorImages were quantified using ImageQuant software (Molecular Dynamics).

**Fluorescence microscopy**

After 3 h serum starvation and 10 min stimulation with 100 nM insulin (where indicated), L6-GLUT4myc cells were fixed for 30 min in 4% formaldehyde in PBS at 4°C followed by a 10 min incubation in 50 mM ammonium chloride in PBS at 20°C to react excess formaldehyde. Cell membranes were then permeabilized by incubation in 0.1% Triton X-100 (v/v) in PBS for 20 min at 20°C and blocked for 30 min at 20°C with 3% goat serum (GibcoBRL) in PBS (w/v). Permeabilized cells were incubated with αSy4 antibody (1:100) (375) and Oregon Green 488 phalloidin (1:1000, from Molecular Probes) for 1 h at 20°C, washed extensively, then incubated with goat anti-rabbit Cy3-conjugated antibody (1:250, from Jackson Immunoresearch) for 1 h at 20°C and washed again. Coverslips were then mounted and fixed onto glass slides using Dako mounting reagent (Dako Corporation, Mississauga, Canada). Images of the resulting fluorescence patterns were collected (bottom of the cells for unstimulated conditions, top of the cells for unstimulated conditions) using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510.
Results

In vitro SNARE binding

Binding assays were performed to quantitate interactions between the three possible binary combinations of SNAP-23, VAMP-2 and syntaxin 4. For the two combinations involving syntaxin 4, a fixed concentration of the GST-syntaxin 4 fusion protein (0.3 μM) was titrated with increasing concentrations of SNAP-23 or VAMP-2. To study the interaction of VAMP-2 with SNAP-23, 0.3 μM GST-VAMP-2 was titrated with increasing concentrations of SNAP-23.

Specific and saturable binding of the soluble SNAREs to the fixed SNAREs was observed for all three binary interactions (Figure 5-1). The syntaxin 4:SNAP-23 interaction was stronger than that of the other two pairs (syntaxin 4:VAMP-2 or SNAP-23:VAMP2), with an apparent half-maximal binding coefficient of 0.76 ± 0.15 μM (Figure 5-1a).

Figure 5-1 Binary binding of SNAP-23, syntaxin 4 and VAMP-2

Increasing concentrations of SNAP-23 (a, c) or VAMP-2 (b) as indicated, were used to titrate fixed 0.3 μM GST-syntaxin 4 (a, b) or fixed 0.3 μM GST-VAMP-2 (c) to saturation. Glutathione-agarose was used to sediment proteins bound to syntaxin 4. The pellets were analyzed by 14% SDS-PAGE and transferred to nitrocellulose. Ponceau S was used to visualize the fixed GST fusion protein constructs (i.e. GST-syntaxin 4 and GST-VAMP-2) while soluble SNAREs were immunoblotted with appropriate antibodies (αSN23.C12 - 1:2,000, αVAMP-2 - 1:1,000). Shown is one experiment representative of three.
Saturation of syntaxin 4 with VAMP-2 was eventually reached at approximately 10 µM VAMP-2 and the resulting apparent half-maximal binding coefficient was 4.4 ± 1.5 µM (Figure 5-1b). SNAP-23 bound to VAMP-2 with an apparent half-maximal binding coefficient of 2.8 ± 0.1 µM (Figure 5-1c). Non-specific binding of VAMP-2 and SNAP-23 to GST alone was found to account for less than 10% of the total soluble SNARE pulled down by GST-syntaxin 4 (results not shown). Since all of the recombinant proteins were raised in bacteria they may not all be in fully native conformations, thus these values represent the minimum binding affinities of the individual SNARE proteins.

SNAP-25 has been shown to enhance the binding of VAMP to syntaxin 1 in vitro (37, 260). It is not known whether SNAP-23, syntaxin 4 and VAMP-2 form a similar complex, although this has been suggested by simple analogy to their neuronal counterparts (87, 274). Through a similar experimental approach as that used for the neuronal SNAREs. we titrated 0.3 µM syntaxin 4 with increasing concentrations of VAMP-2 in the presence of a saturating concentration (2 µM) of SNAP-23. Whereas VAMP-2 binding to syntaxin 4 alone was saturable (Figure 5-1b) we were not able to saturate syntaxin 4 with VAMP-2 in the presence of SNAP-23 (Figure 5-2). VAMP-2 association with syntaxin 4 was observed at high concentrations of VAMP-2, but this coincided with a decrease in the SNAP-23 association with syntaxin 4.

**Figure 5-2** Ternary binding of SNAP-23, syntaxin 4 and VAMP-2

GST-syntaxin 4 (0.3 µM) was incubated in the presence of saturating concentration of SNAP-23 (2 µM) and titrated increasing concentrations of VAMP-2 (c). Glutathione-agarose was used to sediment proteins bound to syntaxin 4. The pellets were analyzed by 14% SDS-PAGE transferred to nitrocellulose. GST-syntaxin 4 levels on the nitrocellulose were visualized with Ponceau S staining, while SNAP-23 and VAMP-2 were detected by immunoblotting with appropriate antibodies (αSN23.C12 - 1:2,000, αVAMP-2 - 1:1,000). Shown is one representative experiment from three.
Coimmunoprecipitation of SNARE proteins

Given the binding affinities measured \textit{in vitro} we explored the possibility that endogenous SNAP-23, syntaxin 4 and VAMP-2 interact \textit{in vivo}. Previous work from our laboratory found that immunoprecipitates of syntaxin 4 from 3T3-L1 adipocytes cell lysates contain VAMP-2 (47). In the present study syntaxin 4 and SNAP-23 were immunoprecipitated sequentially from 3T3-L1 whole cell lysates as described in Experimental Procedures. After SDS-PAGE and transfer to nitrocellulose, the SNAP-23 immunoprecipitate was probed for syntaxin 4 and VAMP-2 while the syntaxin 4 immunoprecipitate was probed for SNAP-23. Syntaxin 4 and VAMP-2 both coimmunoprecipitated with SNAP-23 but, interestingly, SNAP-23 did not coimmunoprecipitate with syntaxin 4 (Figure 5-3).

Figure 5-3 Coimmunoprecipitation of SNARE proteins from 3T3-L1 adipocytes

SNAP-23 or syntaxin 4 were immunoprecipitated (IP) from detergent-solubilized whole cell extracts of 3T3-L1 adipocytes as indicated. Samples were analyzed by 14% SDS-PAGE, transferred to nitrocellulose and immunoblotted (IB) with \(\alpha\)VAMP-2, \(\alpha\)SN23.C12 or \(\alpha\)Sy-4 antibody as described in Methods. The positions of VAMP-2, syntaxin 4 and SNAP-23 are indicated.

SDS-resistant SNARE complexes

A property of the neuronal SNARE complex of SNAP-25, syntaxin 1 and VAMP-2 is its ability to withstand denaturation by SDS (125). It was not known whether non-neuronal SNAREs can form such a complex or whether any one of them can substitute for their cognate non-neuronal SNARE in the complex. We therefore examined the ability of SNAP-23 and syntaxin 4 to participate in SDS-resistant complexes. We determined the ability of both of the
proteins to replace or be replaced by their neuronal counterparts as well as any possible role that GST may play in the formation of complexes by GST-syntaxin proteins.

**Figure 5-4 Only neuronal SNAREs form SDS-resistant complexes *in vitro***

Triton-X-100 solubilized rat brain microsomes (lanes 1-2) were run as a positive control for SDS-resistant complexes. Equimolar concentrations (3.3 μM) of VAMP-2 (lanes 3-10), SNAP-25 (lanes 3-6), SNAP-23 (lanes 7-10), syntaxin 1a (lanes 3-4,7-8) and syntaxin 4 (lanes 5-6, 9-10) were incubated for 2 h at 4°C to allow complexes to form. SDS sample buffer similar in composition to SDS-PAGE buffer (see Methods) was added and samples were either boiled (lanes 2, 4, 6, 8, 10) or not boiled (lanes 1, 3, 5, 7, 9). Samples were analyzed by 12% SDS-PAGE, transferred to nitrocellulose and immunoblotted with syntaxin 1 antibody (lanes 1-2), SNAP-25 antibody (lanes 3-6) or SNAP-23 antibody (lanes 7-10) as described in Methods. 60 kDa bands, indicated in lanes 3-6 by an arrowhead, are non-specific immunoreactive contaminants. Shown is one experiment representative of six.

The results of figure 5-4 confirm previous observations that syntaxin 1 and SNAP-25 form a SDS-resistant complex with VAMP-2. These complexes could be detected in homogenized brain tissue or in mixtures of recombinant proteins. In contrast, however, substitution of either syntaxin 1 or SNAP-25 with their respective non-neuronal counterparts did not allow formation of a SDS-resistant complex (Figure 5-4). In addition, SNAP-23 and syntaxin 4 (or GST-syntaxin 4) together with VAMP-2 do not form a complex resistant to SDS (Figure 5-4). No SDS-resistant complexes were seen in solubilized crude membrane fractions from 3T3-L1
adipocytes (results not shown), possibly due to the low abundance of SNARE proteins in these cells relative to neuronal cells. Experiments identical to these were also probed with primary antibodies to each of the different components of the complexes, to eliminate the possibility of epitope masking by the complex. The results confirmed the formation of SDS-resistant complexes among the neuronal SNAREs but not when syntaxin 4 or SNAP-23 was used.

**In vitro phosphorylation of SNAREs by exogenous kinases**

Protein phosphorylation can regulate certain protein-protein interactions *in vivo*. As the first step in a search for possible phosphorylation events regulating SNARE proteins, we examined the susceptibility of recombinant SNAP-25 and syntaxin 1 and their isoforms to phosphorylation by exogenous PKA, CKII and PKC. GST alone as well as GST-fusion proteins containing full length SNAP-25, full length SNAP-23 or cytoplasmic domains of syntaxins 1 through 4 were exposed to [³²P]γ-ATP in the presence or absence of each of the three kinases. Autoradiography was used to detect the phosphorylated species after SDS-PAGE. Analysis of the amino acid sequences of SNARE proteins by the PROSITE database revealed multiple possible sites for action of CKII, PKA and PKC sites in SNAP-25, SNAP-23 and syntaxins 1 and 4. Using the experimental conditions described in the *Experimental Procedures*, CKII phosphorylated syntaxins 1 and 4 (Figure 5-5a), and PKA phosphorylated syntaxin 4 only (Figure 5-5b). PKC weakly incorporated phosphate into all four syntaxin isoforms, albeit to different extents (Figure 5-5c), but long exposure times were required to detect this phosphorylation. None of the kinases could phosphorylate SNAP-25 or SNAP-23 under the conditions recommended by the kinase manufacturer. When conditions were altered to maximize phosphate incorporation by increasing enzyme, substrate as well as ATP concentration and specific activity (see *Experimental Procedures*), PKC was able to phosphorylate SNAP-25 and to
a lesser extent SNAP-23 (results not shown). Importantly, none of the kinases tested were able to phosphorylate GST.

Figure 5-5 Syntaxin 4 is phosphorylated by CKII, PKA and PKC

GST-SNARE constructs (20 pmol) were incubated with [32P]ATP in the presence (+) or absence (-) of: (a) CKII, (b) PKA, or (c) PKC and in the presence (+) or absence (-) of activators (Ca2+, diacylglycerol and phosphatidylcholine). Phosphorylation reaction products were separated by 10% SDS-PAGE, and phosphorylated bands were detected by autoradiography. Films in (a) and (b) were exposed for 24 h and (c) was exposed for 170 h. The arrowhead in (c) indicates autophosphorylated PKC enzyme. Shown is one experiment representative of three.

Stoichiometry of phosphorylation

To examine the degree of phosphorylation obtained, we determined the stoichiometry of phosphate-incorporation into syntaxin 4 by each of the kinases. Under the conditions used in these experiments, PKA incorporated 3.9 ± 0.4 mole P/mole syntaxin 4; CKII incorporated 0.81
± 0.09 mole P/mole syntaxin 4; and PKC incorporated <0.05 mole P/mole syntaxin 4. A very low phosphorylation was also observed for SNAP-25. Only 0.01 mole P/mole SNAP-25 could be phosphorylated using the high efficiency reaction conditions. Phosphorylation of SNAP-23 was even lower. Therefore, the following binding experiments were performed only with syntaxin 4 phosphorylated by PKA and CKII.

**Figure 5-6 PKA-phosphorylation of syntaxin 4 inhibits SNAP-23 binding**

(a) GST-syntaxin 4 was phosphorylated by PKA as described. Samples of 0.3 μM were incubated with 0.5 or 1.0 μM SNAP-23. Glutathione-agarose was used to sediment proteins bound to syntaxin 4. The pellets were analyzed by 14% SDS-PAGE and transferred to nitrocellulose. GST-syntaxin 4 levels on the nitrocellulose were visualized Ponceau S staining, while SNAP-23 was detected by immunoblotting (1:2,000). Shown is one replicate of one representative. (b) Quantitation of three experiments of three replicates each. Dark bars represent SNAP-23 bound to non-phosphorylated GST-syntaxin 4, and open bars represent SNAP-23 bound to PKA-phosphorylated GST-syntaxin 4 (* Student's test, p<0.05, relative to non-phosphorylated control).
Effects of syntaxin 4 phosphorylation on SNARE binding *in vitro*

To determine the effect of phosphorylation of syntaxin 4 by CKII and PKA on its ability to bind soluble SNAP-23 and/or VAMP-2, assays similar to those used in the titrations described in Figure 5-1 were performed with phosphorylated protein. In these binary interaction assays, the SNAP-23 concentrations of either 0.5 or 1.0 μM were used, while the VAMP-2 concentrations used were 2 and 8 μM. The concentrations were chosen to span the half-maximal binding values for their interactions with unphosphorylated syntaxin 4. In the ternary binding assay 1.0 μM SNAP-23 and 8 μM VAMP-2 were with 0.3 μM (phospho-)GST-syntaxin 4.

*Figure 5-7 PKA-phosphorylation of syntaxin 4 affects only SNAP-23 binding*

(a) GST-syntaxin 4 was phosphorylated by PKA as described. Samples of 0.3 μM were incubated with 1.0 μM SNAP-23 and 8 μM VAMP-2. Glutathione-agarose was used to sediment proteins bound to syntaxin 4. The pellets were analyzed by 14% SDS-PAGE and transferred to nitrocellulose. GST-syntaxin 4 levels on the nitrocellulose were visualized with Ponceau S staining, while SNAP-23 and VAMP-2 were detected by immunoblotting (1:2,000 and 1:1,000 respectively). Shown is one replicate of one representative experiment. (b) Quantitation of three experiments of three replicates each. Dark bars represent soluble SNARE protein bound to non-phosphorylated GST-syntaxin 4, and open bars represent soluble SNARE protein bound to PKA-phosphorylated GST-syntaxin 4 (* Student's t-test, p<0.05, relative to non-phosphorylated control).
CKII phosphorylation did not affect the ability of syntaxin 4 to bind SNAP-23 or VAMP-2 in either binary or ternary combinations (results not shown, p>0.05). On the other hand, PKA-phosphorylated syntaxin 4 consistently bound 30% less SNAP-23 than non-phosphorylated syntaxin 4, in both binary (Figure 5-6) and ternary (Figure 5-7) assays. In contrast, the binding of VAMP-2 did not appear to be affected by PKA phosphorylation in either the binary assay (results not shown, p>0.05) or the ternary assay (Figure 5-7).

**SNAP-23 specifically interacts with the cytoskeleton**\(^{13}\)

Recently, it was reported that the endogenous SNAP-23 of mast cells is able to relocate intracellularly, possibly due to association with cytoskeletal elements (110). Triton X-100 insolubility is frequently used to predict interactions with cytoskeletal elements (55, 110). To examine if SNAP-23 partitions with cytoskeletal elements in 3T3-L1 adipocytes, Triton X-100-soluble and -insoluble fractions were purified from isolated crude membrane fractions, and the presence of SNAP-23 was determined by immunoblotting. The results indicate that endogenous SNAP-23 partitions into both Triton X-100-soluble and insoluble components (Figure 5-8). Analysis of the total protein yields in each fraction revealed that approximately 5% of the membrane-bound SNAP-23 originally isolated was recovered in the Triton-insoluble fraction. As expected, this fraction was shown to contain actin (Figure 5-8) and is therefore likely to contain the insoluble cytoskeleton. Actin present in the soluble fraction is likely to represent the non-filamentous form of this protein. In contrast to SNAP-23, the SNAREs syntaxin 4 and VAMP-2 were found exclusively in the Triton-soluble fractions (Figure 5-8), highlighting that the tight SNAP-23 association with the Triton-insoluble fraction is a particular property of this SNARE.

\(^{13}\) This set of experiments was done in partnership with Karen Yaworsky, a M.Sc. student in the laboratory at the time and are used with her permission.
Figure 5-8 SNAP-23, but not VAMP-2 or syntaxin 4, associates with the cytoskeleton

Crude membranes were prepared from serum-starved 3T3-L1 adipocytes as described in Methods. Membranes were then solubilized in Triton X-100-containing buffer and centrifuged to pellet insoluble material (I), leaving the soluble proteins (S) in the supernatant. Proteins in the soluble and insoluble fractions were then separated by 13% SDS-PAGE and analyzed by immunoblotting. Shown are representative blots from four independent experiments. The entire pellet of insoluble material was run next to 1/20th of the total protein from the soluble fraction.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight (kDa)</th>
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<tbody>
<tr>
<td>Actin</td>
<td>-</td>
</tr>
<tr>
<td>Syntaxin 4</td>
<td>- 35 kDa</td>
</tr>
<tr>
<td>SNAP-23</td>
<td>- 28 kDa</td>
</tr>
<tr>
<td>VAMP-2</td>
<td>- 16 kDa</td>
</tr>
</tbody>
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SNAREs concentrate in insulin-induced membrane ruffles

Rearrangement of the actin cytoskeleton is believed to play a significant role in directing the spatial arrangement of signalling molecules involved in GLUT4 traffic as well as of GLUT4 itself. Recent work from our laboratory has demonstrated that certain molecules, including GLUT4, move specifically to sites where insulin is actively causing the rearrangement of the underlying actin cytoskeleton (171). In a separate study, work from our laboratory showed that VAMP-2 (271) and SNAP-23 (Z. Khayat, personal communication) are among the proteins that move to these sites of actin rearrangement. In order to see if syntaxin 4 also translocates to these ruffles, L6-GLUT4myc myoblasts were stimulated with or without 100 nM insulin and stained with the filamentous actin dye phalloidin as well as syntaxin 4 antibodies followed by anti-rabbit secondary antibodies. Examination of the cells by confocal fluorescent microscopy revealed that in unstimulated cells syntaxin 4 is distributed in a punctate pattern throughout the cells with some concentration at leading edges (Figure 5-9a). Insulin stimulation of the cells causes syntaxin 4 to move to sites where actin is being rearranged (Figure 5-9b), in conjunction with its other SNARE partner.
Figure 5-9 Syntaxin associates with insulin-induced membrane ruffles

Serum starved L6-GLUT4myc myoblasts were incubated in the absence (a) or presence (b) of 100 nM insulin for 10 min. The cells were then fixed, permeabilized and labelled for filamentous actin (green) and syntaxin 4 (red). Areas where the two signals intersect appear as yellow. The cells in (b) were imaged near the top of the cell where the ruffles are forced up to. As there was no actin staining visible near the tops of unstimulated cells, the confocal image in (a) was collected from near the bottom of the cells.

PKB phosphorylates syntaxin 4 in vitro

The work illustrated in Figure 5-5 indicated that syntaxin 4, and to a lesser extent SNAP-23, are phosphorylated in vitro by several kinases, however none of them is thought to play a significant role in insulin signalling. In order to explore if these SNAREs are phosphorylated by kinases known to mediate insulin signals we examined the susceptibility of SNAP-23, syntaxin 4 and VAMP-2 to phosphorylation by immunopurified PKB\(\alpha\) and PKC\(\zeta\). Using methods established previously in our laboratory (see Experimental Procedures), recombinant syntaxin 4, SNAP-23 and VAMP-2 were used as in vitro substrates for immunopurified PKB\(\alpha\) and PKC\(\zeta\). The results revealed that syntaxin 4 is phosphorylated by both kinases\(^{14}\) (Figure 5-10). PKB phosphorylation of syntaxin 4 reproducibly increased with insulin stimulation of the cells prior to isolation of the kinase. Conversely, while PKC\(\zeta\) immune complexes were able to phosphorylate syntaxin 4, there was no effect of insulin on this phosphorylation. Neither GST-VAMP-2 nor GST-SNAP-23 nor GST alone were phosphorylated by PKB or PKC\(\zeta\) (Figure 5-10).

\(^{14}\) Romel Somwar, a Ph.D candidate in our laboratory performed the first replicate of this experiment using substrates prepared by the candidate, who did the remaining two replicates.
**Figure 5-10 PKBα and PKCζ phosphorylation of SNAREs**

<table>
<thead>
<tr>
<th>Insulin</th>
<th>PKBα</th>
<th>PKC</th>
<th>Substrate</th>
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<tbody>
<tr>
<td>-</td>
<td>GST</td>
<td>GST</td>
<td>Sn N2 V2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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- 69 kDa
- 43 kDa
- 29 kDa

Recombinant syntaxin 4(Sn 4), GST-SNAP-23 (GST-SN23), GST-VAMP-2 (GST-V2) and GST alone (50 pmol each lane) were incubated with 32P-γ-ATP and PKBα (left gel) or PKCζ (right gel) immunoisolated from cells treated with (+) or without (-) 100 nM insulin. Phosphorylation reaction products were separated by 10% SDS-PAGE, and phosphorylated bands were detected by PhosphorImager. Shown is one experiment representative of three.

**Insulin does not cause SNARE phosphorylation in vivo**

The evidence presented thus far suggests: 1) that SNAREs can act as substrates of serine/threonine kinases and 2) that these phosphorylations are potential regulators of SNARE-SNARE interactions. However, the phosphorylations reported so far all occur *in vitro* and there is no evidence that they occur *in vivo*. In order to demonstrate a physiologically significant SNARE phosphorylation we immunoprecipitated VAMP-2, SNAP-23 and syntaxin 4 from fully differentiated 3T3-L1 adipocytes loaded with 32P-γ-phosphate. PhosphorImager analysis of the SDS-PAGE-resolved immune complexes revealed that all three proteins possess a background level of phosphorylation but that this level is not altered in cells treated with insulin (Figure 5-11). Immunoblotting of the same samples suggests that differential protein loading is not the explanation for a lack of difference between the stimulated and unstimulated signals. However, if a phosphorylation event were occurring on a subpopulation of any one of these proteins (i.e. the
VAMP-2 on GLUT4 vesicles) then those changes might be masked in the context of the entire cellular complement of that protein.

Figure 5-11 Neither insulin nor isoproterenol cause phosphorylation of SNAREs in vivo
Syntaxin 4, VAMP-2 and SNAP-23 were immunoprecipitated from 3T3-L1 adipocytes loaded with ^32P-orthophosphate as described in Experimental Procedures. The immune complexes were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes and exposed to a PhosphorImager screen.

Discussion

It is well established that SNARE proteins are required for the docking and fusion of vesicles with their target membrane in a variety of organisms and tissues (for reviews see (38, 87, 274)). The observation that neuronal isoforms of SNARE proteins can assemble into a number of multimeric complexes has led to the suggestion that the stepwise progression of SNARE proteins from binary to ternary to 7S and finally to 20S complexes precedes the fusion of the vesicle with the target membrane as discussed in Chapter 2 (282, 314). The natural extension of such a hypothesis is that individual SNARE isoforms have cognate partners that form analogous complexes leading to fusion of specific vesicle classes with their appropriate target membranes. In other words, pairing between specific sets of SNAREs defines vesicle targeting specificity. As discussed in Chapter 2, this postulate was originally called the SNARE hypothesis (282, 314) and recent work from the same laboratory, using an in vitro assay of vesicle fusion, has demonstrated that this hypothesis is probably true. Examination of all the t-SNAREs in yeast (where the genome is sequenced and thus all t-SNAREs are known) revealed
vesicle fusion, has demonstrated that this hypothesis is probably true. Examination of all the t-SNAREs in yeast (where the genome is sequenced and thus all t-SNAREs are known) revealed that only those in the appropriate locations allow fusion with vesicles carrying v-SNAREs destined for those locations (219). Many lines of evidence support the notion that SNAP-23 and syntaxin 4 are t-SNARE partners for the broadly expressed v-SNARE VAMP-2, including their expression in compartments known to fuse with each other and their ability to bind one another in binary combinations (47, 248, 273, 375). Furthermore, the results presented here and work from other laboratories has shown that these proteins are capable of forming binary complexes (37, 47, 260, 273).

We show here that the binary complexes between SNAP-23, VAMP-2 and syntaxin 4 form in the same rank order of affinity as the neuronal isoforms. The interaction between SNAP-23 and syntaxin 4 was the strongest of the three pairs (apparent half-maximal binding coefficient of 0.76 ± 0.15 μM), the binding between SNAP-23 and VAMP-2 was found to be of intermediate affinity (apparent half-maximal binding coefficient value of 2.8 ± 0.1 μM), and the binding of VAMP-2 to syntaxin 4 was the weakest. However, in contrast with previous results in which binding of VAMP to syntaxin 1 could not be saturated (260), we were able to saturate syntaxin 4 with VAMP-2 (apparent half-maximal binding coefficient of 4.4 μM • 1.5). In support of the strong binary interactions we observed in vivo, we also show that SNAP-23 can coimmunoprecipitate both VAMP-2 and syntaxin 4. Although syntaxin 4 immunoprecipitates were shown to contain VAMP-2 (47) we found that these immunoprecipitates do not contain SNAP-23 (Figure 5-3). This is not due to a lower abundance of SNAP-23 relative to other SNAREs in 3T3-L1 cells since it has been demonstrated that SNAP-23 is approximately 3 times more abundant that either syntaxin 4 or VAMP-2 on a whole-cell basis (131). However, consistent with the in vitro results, the lack of SNAP-23 in syntaxin-4 immunoprecipitates could
be due to displacement of SNAP-23 by VAMP-2 (see below). It is not likely to be due to selectivity of the immunoprecipitating antibody for free syntaxin 4 since the antibody used to immunoprecipitate syntaxin 4 is a polyclonal one raised against the full cytoplasmic domain of syntaxin 4.

A further unexpected finding of our studies is that we could not observe ternary complex formation among the non-neuronal combination of SNARE proteins, in either non-denaturing binding assays or SDS-resistance assays. Not only was VAMP-2 unable to form a SDS-resistant complex with SNAP-23 and syntaxin 4, but also neither of these two proteins on their own were able to replace SNAP-25 or syntaxin 1 in the neuronal SDS-resistant complex. Whereas lack of SDS resistance does not directly prove lack of ternary complex formation, it does suggest that if the complex exists the forces that keep the proteins together are much weaker than those involved in the tripartite complex of the neuronal isoforms. Furthermore, when increasing concentrations of VAMP-2 were added to syntaxin 4 and SNAP-23, VAMP-2 association with syntaxin 4 resulted in decreased SNAP-23 association. These results suggest that SNAP-23 and VAMP-2 may compete for a similar site on syntaxin 4 or, alternatively, that excess VAMP-2 removes unbound SNAP-23 from its equilibrium with syntaxin 4. While SNAP-23 immunoprecipitates contained both VAMP-2 and syntaxin 4, this does not constitute evidence of a ternary SNARE complex, since the stoichiometry of the three SNAREs in the immunoprecipitate is not known. It is conceivable that the immunoprecipitate contains binary complexes of SNAP-23:VAMP-2 and SNAP-23:syntaxin 4.

The lack of evidence for ternary complex formation in vitro among a non-neuronal combination of SNARE isoforms is puzzling and raises some interesting possibilities. The simplest explanation is that these three proteins do not form a ternary complex in vivo, and that pairwise interactions are the only requirement for docking and fusion steps involving these
SNAREs. However, based on recent evidence from Rothman’s laboratory using their in vitro fusion assay (384) this is unlikely since it appears that four helices contributed by at least three different proteins is an absolute requirement for fusion (94, 251). A second possibility is that additional factors such as NSF or αSNAP are required for the formation of a stable complex. Indeed, when a membrane extract of rat adipocytes was incubated with myc-epitope-tagged NSF and αSNAP, the endogenous syntaxin 4 and VAMP-2 sedimented upon myc-epitope immunoprecipitation (346), and the immunoprecipitate was later shown to contain SNAP-23 (320). While the presence of all three proteins in an immune complex is not proof of a ternary complex another group was able to isolate a stable complex containing recombinant SNAP-23, VAMP-2 and syntaxin 4 using size exclusion chromatography (395). In support of our results this complex was also not stable in SDS and exhibited a melting point temperature much lower than that reported for other SNARE complexes including the neuronal complex of syntaxin 1a, VAMP-2 and SNAP-25 (395). A third possible explanation for the lack of a ternary complex in our assay is that the ternary complex forms but its dissociation rate is too rapid to be detected. To this end experiments using surface plasmon resonance, a technique that is capable of detecting much more transient interactions, were able to detect an SDS-resistant complex between SNAP-23, VAMP-2 and syntaxin 4, albeit using much lower concentrations of SDS (275).

The reasons for the different stabilities of the neuronal SNARE complex and the complex containing SNAP-23, syntaxin 4 and VAMP-2 are not clear, but we can consider some possibilities. One major difference between regulated fusion events in neurons and in non-neural cells is their time course, i.e., fusion of synaptic vesicles in neurons occurs within fractions of a millisecond while exocytosis in many other cell types occurs much more slowly (150). The rapidity of synaptic vesicle exocytosis is thought to occur because vesicles are docked at the plasma membrane in a pre-fusion state requiring only a calcium influx to complete the fusion
process. In contrast, in many of these non-neuronal cell types regulated exocytosis may be controlled in part by rate-limiting steps prior to the docking/fusion process (52). For example, in the chromaffin cell, depolymerization of cortical actin appears to be necessary to facilitate exocytosis (369) and in the case of GLUT4 translocation to the plasma membrane the rate-limiting step may be formation of the exocytic vesicle (see Chapter 1). In some cases, docking/fusion steps may occur constitutively after the regulatory signal, in which case the ternary complex would not have to wait for a fusion 'trigger' as the neuronal complex does, and may, therefore, not have to be particularly stable.

In such a case, cellular regulation of the assembly of the binary complexes may be important in the control of exocytosis. Protein phosphorylation has been established as a universal method of regulating protein-protein interactions. Some SNARE proteins have been identified as targets of various kinases both in vivo (54, 85, 100, 167, 300, 400) and in vitro (135, 279). In this study we are the first to demonstrate that syntaxin 4 is an efficient in vitro substrate for PKA and CKII. No other syntaxin tested was phosphorylated by PKA. Syntaxin 1 was phosphorylated by CKII and SNAP-25 was phosphorylated by PKC to low levels, consistent with previous observations (135, 300). Hirling and Scheller (135) reported that PKA can also phosphorylate SNAP-25, albeit to a very low extent, so it is possible that this level of phosphorylation was too low to be detected under the conditions we used. Our results further show that SNAP-23 cannot be phosphorylated in vitro by either PKA or CKII, and only minimally by PKC.

Importantly, we have identified a functional consequence of syntaxin 4 phosphorylation by PKA. PKA-phosphorylated syntaxin 4 cannot bind the same amount of SNAP-23 at equilibrium that non-phosphorylated syntaxin 4 can. SNAP-23 binding by syntaxin 4 is consistently decreased by 30% upon phosphorylation by PKA. Such a shift in the equilibrium
binding constant could significantly alter the ability of proteins to interact in vivo where protein concentrations may be limiting and kinases may be temporally and spatially regulated. This was the first evidence that phosphorylation of syntaxin 4 had a functional consequence on its interaction with SNAP-23. Since the publication of this data other investigators have reported similar findings (54, 279).

PKA is a serine/threonine kinase that requires cyclic adenosine monophosphate (cAMP) for activity. This requirement for cAMP puts PKA downstream of adenylate cyclase that in turn can be activated by numerous pathways, most notably through activation of heterotrimeric G-protein-coupled receptors such as the β-adrenergic receptor. Activation of the β-adrenergic receptor by adrenaline itself or the adrenomimetic compound isoproterenol can have counter-regulatory effects on insulin’s ability to stimulate glucose uptake. Pretreatment of cells with isoproterenol can inhibit insulin-stimulated glucose transport in rat adipocytes (42, 159, 204). In the presence of isoproterenol, insulin appears to be able to mobilize GLUT4 proteins to the plasma membrane as determined by Western blotting of plasma membrane fractions and by membrane-invasive, glucose transporter-specific labeling (42, 159, 204). However, GLUT4 does not appear to translocate to the plasma membrane as determined by exofacial labeling (81, 364). One possible explanation of this phenomenon is that insulin stimulates the delivery of the GLUT4 vesicles to the plasma membrane, but upon arrival there they are unable to fuse with it. Isoproterenol may have caused PKA to phosphorylate syntaxin 4, resulting in blockage of a syntaxin 4:SNAP-23 interaction that is required for vesicle fusion with the plasma membrane.

While the data presented in figures C2-6 and C2-7 present a potential method for explaining how isoproterenol can inhibit insulin-stimulated glucose uptake we found no evidence that the phosphorylation state of syntaxin 4 was changed by stimulation with insulin or isoproterenol (figure 5-11). This suggests that neither PKA nor PKBα and PKCζ, which also
phosphorylated syntaxin 4 \textit{in vitro}, are capable of phosphorylating syntaxin 4 \textit{in vivo}. However, while syntaxin 4 may not be regulated by direct phosphorylation its location within the cell is regulated by insulin since it moves preferentially to sites of active actin rearrangement in response to insulin. This movement may be an indirect effect where SNAP-23 mediates the interaction but this possibility requires further experimental analysis.

The observation that SNAP-23 is found in fractions enriched in actin (Figure 5-8) raises the possibility that an interaction between SNAP-23 and the cytoskeleton could facilitate the positioning of the incoming vesicles, improving their access to either insulin-signaling molecules and/or to the fusion machinery. Indeed, members of that fusion machinery (i.e. SNAP-23, syntaxin 4 and VAMP-2) can be seen in actin ruffles by fluorescence microscopy even though only SNAP-23 was found in the actin-enriched biochemical fractions. SNAP-23’s association with cytoskeletal elements is born out by studies in other systems. In mast cells SNAP-23 is found in ‘plasma membrane lamellipodia-like projections’ (110) and in fibroblasts vimentin filaments act as a reservoir of SNAP-23 to supply the plasma membrane with fusion machinery (77). Vimentin is actually an intermediate filament protein while lamellipodia are actin-based structures but both studies, in addition to the data presented here, provide evidence that SNAP-23 may work with various cytoskeletal proteins to direct vesicle traffic.

In conclusion we have detected and quantitated binary interactions between SNAP-23, syntaxin 4 and VAMP-2 but have found no evidence for a ternary complex of these three proteins under conditions which promote formation of stable ternary complexes among the neuronal isoforms. We have also shown that syntaxin 4, when phosphorylated by PKA, is deficient in its ability to bind SNAP-23. However, syntaxin 4 is not phosphorylated \textit{in vivo} by insulin or isoproterenol stimulation but does localize specifically to sites of actin rearrangement.
Chapter 6 – Role of SNAP-23 in insulin-dependent GLUT4 translocation

Summary

The acute stimulation of glucose uptake by insulin in fat and muscle cells is primarily the result of translocation of GLUT4 facilitative glucose transporters from an internal compartment to the plasma membrane. The final step of fusing the exocytic GLUT4 vesicles with the plasma membrane involves VAMP-2 and syntaxin 4, each contributing one helix to the four helix bundle required for fusion. Thus, the SNARE hypothesis predicts that two further helices must be contributed by another molecule(s). The most likely candidate in this case is SNAP-23 since it is expressed along with syntaxin 4 in the plasma membrane of fat and muscle cells and it binds to both syntaxin 4 and VAMP-2 (see Chapter 2) but prior to this study it was unknown if SNAP-23 participated in insulin-stimulated GLUT4 traffic.

The purpose of this study was to explore the participation of SNAP-23 in insulin-regulated GLUT4 movement to the plasma membrane. Clostridial neurotoxins have proven to be valuable tools for the study of SNARE function in neuronal systems, however many non-neuronal SNAREs, including rodent forms of SNAP-23, are not affected by these proteases (50, 205, 284). Therefore, other approaches had to be developed to perturb SNARE proteins in non-neuronal systems. Here we have investigated the role of the putative t-SNARE SNAP-23 in GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes by perturbing its function using full-length protein and a peptide that mimics the carboxy-terminal region of SNAP-23 as well as an antibody raised against a smaller carboxy-terminal peptide.

**Experimental procedures**

**Fusion proteins and antibodies**

Polyclonal antibodies to GLUT4 (374) and SNAP-23 (termed αSN23.c12) were described previously (389). Fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG, Cy3-conjugated goat anti-rabbit IgG and rhodamine-conjugated dextran were obtained from Molecular Probes, Inc. (Eugene, OR). GST (glutathione S-transferase) and N-terminal-fused GST-SNAP-23 were prepared as described previously in Chapter 5. Horseradish peroxidase-conjugated protein A secondary antibodies were purchased from Bio-Rad. A synthetic peptide corresponding to the carboxy-terminal 20 amino acids of SNAP-23 (DTNDRIDIANARAKKLIDS, termed SN23Pep) was obtained from the Biotechnology Service Centre (Hospital for Sick Children, Toronto, ON). An unrelated control peptide (CVRRASEPGNRKGLGNEK, termed UnrelPep) was a generous gift of Dr. Sergio Grinstein (Hospital for Sick Children).

**GST-syntaxin 4/SNAP-23 binding assay**

To assay the ability of SN23Pep to interfere with binding between SNAP-23 and GST-Syntaxin 4, 0.3 μM GST-syntaxin 4 was incubated with 1 μM SNAP-23 and either 200 μM SN23Pep or 200 μM UnrelPep under the same conditions described for *in vitro* binding assays described in Chapter 5 (2 h, 4°C). The protein bound to GST-syntaxin 4 was then pelleted with the agarose beads and solubilized in SDS sample buffer. Resolution of the proteins by SDS-PAGE followed by immunoblotting (for SNAP-23) or Ponceau S staining (for GST-syntaxin 4) allowed detection of the precipitated proteins.
Tissue culture.

3T3-L1 adipocytes were grown and differentiated according to previously published protocols (374). Briefly, the cells were maintained as fibroblasts in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum (CS) and 1% (v/v) antibiotic/antimycotic solution. 3T3-L1 fibroblasts were induced to differentiate into adipocytes by replacing the DMEM/CS with DMEM supplemented with 10% FBS, 1% (v/v) antibiotic/antimycotic solution and 0.25 μM dexamethasone, 0.5 mM 1-methyl-3-isobutylxanthine and 10 μg/mL porcine insulin. After four days the medium was changed to DMEM/FBS with 10 μg/mL insulin for an additional two days then replaced every two days with DMEM/FBS. Adipocytes were used in experiments between 11 and 14 days after initiation of differentiation. Cells were passaged as fibroblasts by washing with PBS, incubating with 0.05% (w/v) trypsin to lift cells off and diluted into DMEM/CS 3T3-L1 for replating.

Single cell microinjection and plasma membrane lawns

Coverslips covered with 3T3-L1 adipocyte monolayers were transferred to coverslip chambers (Medical Systems Corp. Greenvale, NY) containing 1 mL HPMI medium. Cells in a marked, 1 mm² region of the coverslip were microinjected using an Eppendorf microinjection unit (Micromanipulator 5171 and Transjector 5246) mounted on a Nikon fluorescence microscope. Custom boroisilicate microinjection pipettes (World Precision Instruments) were pulled using a Sutter Instruments Flaming/Brown micropipette puller (P-97). Approximately 90% of the cells in the marked region (~100 cells) were microinjected with 20 μM GST, 20 μM GST-SNAP-23, 0.1 μg/μL αSN23.c12, 2 mM SN23.c20 or 200 mM Unrel or 0.1 μg/μL irrelevant IgG in a solution containing 1.1 μg/μL rhodamine-dextran (Mr 10,000), 110 mM potassium acetate, 10 mM HEPES (pH 7.2) and 1 mM EDTA. The volume microinjected was
about 1/10 of the cell volume. This calculation is based on estimates of the volume of buffer delivered by the microinjector into oil containing 1 μm-diameter latex beads for size comparison (113).

After microinjection cells were incubated in DMEM for 2 hours followed by a 15 min stimulation with 100 nM insulin, where indicated. Plasma membrane lawns (sheets) were prepared by first swelling the cells in a hypotonic solution (23 mM KCl; 10 mM HEPES, pH 7.5; 2 mM MgCl₂; 1 mM EGTA) and then disrupting their membranes in sonication buffer (20 mM HEPES, pH 7.4; 100 mM KCl; 2 mM CaCl₂; 1 mM MgCl₂, 1 μg/mL leupeptin; 10 μg/mL pepstatin; 2 mM PMSF). Disruption was achieved by forcing sonication buffer over the cells using a p1000 Gilson Pipetteman. Sonication buffer was then removed and the resulting lawns were washed twice with more sonication buffer, fixed for 10 min in 3% formaldehyde on ice, quenched for 5 min with 50 mM NH₄Cl in PBS at room temperature and blocked for 30 min with 5% goat serum in PBS. The lawns were then incubated with anti-GLUT4 antiserum (1:150 dilution) for 60 min at room temperature and, after three washes with PBS, incubated for a further 50 min at room temperature with Cy3-conjugated goat anti-rabbit IgG (1:150 dilution). After three more washes with PBS the coverslips were mounted using Dako fluorescent mounting reagent. Fluorescent images of lawns were obtained using a Leica inverted fluorescence microscope (model DM IRB) and quantitated and analyzed using NIH Image software (NIH, Bethesda, MD). By this approach, over 90% of the cells in the marked area can be microinjected. Any uninjected cells within the lawns quantitated as 'microinjected' in these experiments will result in underestimation of the effects of the microinjected test material. Hence, all effects reported for microinjected material, whether increasing or decreasing GLUT4 content, may be underestimated by about 10%.
It has been reported that insulin can increase the number of 3T3-L1 adipocytes that respond to insulin by translocating GLUT4 (211). We therefore assessed whether the microinjected materials tested in the present study affect the number of responding cells. Insulin-response was operationally defined as any surface GLUT4 signal > X - σ, where X and σ are the mean and standard deviation of the non-injected, insulin-treated cell population.

**Preparation of crude membrane and cytosol fractions**

Cytosol and crude membrane fractions from 3T3-L1 cells were prepared as described in Chapter 5. For preparation of Triton X-100-soluble and -insoluble fractions, entire crude membrane pellets from two wells of a 6-well plate of fully differentiated adipocytes were resuspended in Lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 0.25% deoxycholate, 1.0% Triton X-100, 10 mM NaPPI, 100 mM NaF, 2 mM EDTA, 10 μg/mL aprotinin, 2 μM leupeptin, 2 μM peptatin A, 2 mM PMSF, 1 mM NaVO4. After vigorous mixing these suspensions were centrifuged at ~3,000 x g for 10 min. The pellet of this step was considered to be the Triton-insoluble fraction and the supernatant the Triton-soluble fraction.

**Glucose transport in SLO-permeabilized cells**

The ability of insulin to stimulate glucose transport in streptolysin O (SLO)-permeabilized 3T3-L1 adipocytes was measured as described previously (373). The concentration of SLO used (0.675 μg/mL) creates pores large enough to allow immunoglobulin molecules to enter the cells (375). Cell monolayers were first washed three times with potassium glutamate buffer (termed KGB, 138 mM glutamic acid, 20 mM HEPES, 8 mM MgCl2, 0.285 mM CaCl2, 1 mM EGTA) and then incubated for 5 min at room temperature with SLO solution (KGB containing 0.675 μg/mL SLO, 1 mM ATP, 20 μg/mL creatine phosphokinase, 8 mM...
phosphocreatine) for 5 minutes at 37°C. Following permeabilization the SLO solution was removed and replaced with KGB and either 0.1 mg/mL αSN23.c12 or irrelevant IgG or 20 μM GST-SNAP-23 or GST and incubated for 15 min at 37°C. Insulin (100 nM), where indicated, was added for a further 15 min in the presence of the proteins or antibodies. Stock transport solution (final concentrations: 10 μM [3H]2-deoxyglucose (1 μCi/mL), 2 μM [14C]sucrose (0.2 μCi/mL)) was then added directly to the wells and incubated for a further 5 min at room temperature. All steps after and including permeabilization were performed in the presence of an ATP-regeneration system (ATP, creatine phosphokinase and phosphocreatine). Cells were then lysed in 0.05M NaOH and associated radioactivity measured by scintillation counting. The spillover of 14C into the 3H recording channel was subtracted. Permeabilization with SLO increased the diffusional component of glucose uptake by four to five fold, to represent approximately 25% of the total uptake. Facilitative transport was calculated by subtracting the diffusion-dependent 14C signal from the amount of [3H]2-deoxyglucose associated with the cells (375). Statistical analysis was done using ANOVA.
Results

Figure 6-1. Microinjection of SNAP-23 antibodies reduces GLUT4 incorporation into the plasma membrane

Approximately one tenth of the cell volume of 0.1 μg/μL αSN23.c12 or irrelevant IgG were microinjected into ~90% of all cells in an outlined region of the coverslip. The coverslips were then serum-starved for 2 hours and incubated a further 15 min in the absence (a, left panels) or presence (a, right panels) of insulin. Plasma membrane lawns were prepared as described and stained for GLUT4. Indirect immunofluorescence images of GLUT4 on plasma membrane lawns from non.injected (a, top row) and injected (a, second and third rows) cells were collected. Representative images from one of five experiments are shown. (b) Images such as those seen in (a) were digitally quantitated by measuring average intensities for individual lawns. Values from individual experiments were averaged and normalized to basal values. Values represent mean ± SE of the effect of insulin relative to its corresponding basal control, of five independent experiments. Asterisk (*) denotes data which are significantly different from either control (p < 0.05, ANOVA).

Antibodies to SNAP-23 inhibit GLUT4 translocation

The participation of the endogenous SNAP-23 in insulin-dependent association of GLUT4 with the cell membrane was first assessed by introducing into 3T3-L1 adipocytes
antibodies raised to a hemocyanin-linked peptide comprising the C-terminal 12 amino acids of SNAP-23 (termed αSN23.c12). The antibody, in an isotonic potassium acetate buffer, was microinjected into 3T3-L1 adipocytes and its effect on GLUT4 content in membrane lawns was measured 90-120 min later (see Experimental Procedures). Microinjection of αSN23.c12 or unrelated rabbit IgG did not alter the amount of plasma membrane GLUT4 in the basal state (Figure 6-1a, top panels). In contrast, microinjection of αSN23.c12 diminished the insulin-stimulated increase in GLUT4 on the membrane lawns (Figure 6-1a, middle bottom panel). Unrelated IgG did not have any effect on insulin action (Figure 6-1a, right bottom panel). Digital quantitation of lawns from five independent experiments confirmed that, indeed, αSN23.c12 causes a statistically significant decrease in insulin-stimulated GLUT4 incorporation into the membrane (Figure 6-1b).

**GST-fused SNAP-23 enhances insulin-stimulated GLUT4 translocation**

To more directly prove the participation of SNAP-23 in GLUT4 organelle fusion, we tested the effect of exogenous full length SNAP-23 in this process. The protein is naturally soluble but attaches to membranes via palmitoylation and association with other proteins such as syntaxin-4. Therefore, it was conceivable that introduction of excess full-length SNAP-23 might increase membrane fusion events if free syntaxin-4 and incoming vesicles are present. Recombinant GST-SNAP-23 was able to interact with the cytosolic domains of VAMP-2 and syntaxin-4 *in vitro* in a manner previously demonstrated with recombinant SNAP-23 binding to VAMP-2 and GST-syntaxin-4 (*Chapter 5 and (91)).

GST-SNAP-23 was microinjected into a clearly identified area of serum-deprived 3T3-L1 adipocytes. Based on the volume microinjected and the calculated cell volume, the final concentration of SNAP-23 introduced may reach 2 µM. The cells were incubated in culture
medium for 90 min, followed by stimulation with 100 nM insulin for 15 min. Membrane lawns were immediately generated as described in *Experimental Procedures*, and GLUT4 was detected on these lawns by indirect immunofluorescence. The signals from cells in the area defined for microinjection were compared with those of adjacent, non-injected cells. As observed previously (373), insulin caused an increase in GLUT4 labeling on plasma membrane lawns from non-injected cells (Figure 6-2). Microinjection of GST-SNAP-23 had no effect on the amount of GLUT4 detected in the basal state, compared with adjacent, non-injected cells. Digital analysis of four independent experiments of unstimulated (basal) cells showed that microinjection of GST-SNAP-23 did not alter the GLUT4 signal relative to vicinal-non-injected cells. On average, 36 lawns were quantitated within each experiment. The GLUT4 signal in the non-injected cells was assigned a value of 1.00 within each experiment, and the effect of GST-SNAP-23 was calculated relative to this value. In the four experiments, the microinjected cells had a GLUT4 signal of 0.99 ± 0.14 (mean ± S.E.). Therefore, GST-SNAP-23 did not affect the basal amount of GLUT4 present at the plasma membrane (Figure 6-2).

We then examined the effect of insulin on GLUT4 levels on membrane lawns. The GLUT4 signal in the basal state was assigned a value of 1.00 in each experiment, and the effect of insulin was calculated in relative units. In four independent experiments with non-injected cells, insulin caused a 2.53 ± 0.07 fold (mean ± S.E.) increase in GLUT4 over basal values. In lawns from cells microinjected with GST-SNAP-23, the insulin response rose to 3.20 ± 0.30 fold (mean ± S.E.) relative to unstimulated, microinjected cells (Figure 6-2). This gain of 26% in insulin response caused by GST-SNAP-23 was statistically significant vis-á-vis both non-injected and GST-injected cells (p < 0.05, ANOVA) (Figure 6-2).
**Figure 6-2. Microinjection of exogenous SNAP-23 protein improves GLUT4 incorporation into the plasma membrane**

Approximately one tenth of the cell volume of 20 μM GST-SNAP-23 or GST were microinjected into ~90% of all cells in an outlined region of the coverslip. The coverslips were then serum-starved for 2 hours and incubated a further 15 min in the absence or presence of insulin. Plasma membrane lawns were prepared as described and stained for GLUT4. Indirect immunofluorescence images of GLUT4 on plasma membrane lawns from non-injected and injected cells were collected. Intensity of GLUT4 staining in plasma membranes was digitally quantitated by measuring average intensities for individual lawns. Values from individual experiments were averaged and normalized to basal values. Values represent mean ± SE of the effect of insulin relative to its corresponding basal control, of four independent experiments. Asterisk (*) denotes data which are significantly different from either control (p < 0.05, ANOVA).

The above results are based on the averaged GLUT4 signal from multiple lawns per field. To assess whether GST-SNAP-23 could have changed the number of insulin-responding cells, the signal from each lawn was counted as positive or negative using the cut-off value described under *Experimental Procedures*. By this analysis, 95.6 ± 3.1% (mean ± S.E.) of cells microinjected with GST-SNAP-23 were found to respond to insulin, compared to 84.6 ± 2.3% of non-injected cells. The difference between both values was statistically significant at the p < 0.05 level (Student’s t-test). However, the number of responding cells increased by only 10%, whereas the average gain in GLUT4 per lawn was 26%. Taken together, these results suggest that not only does microinjection of GST-SNAP-23 increase the number of responding cells, it further stimulated the magnitude of the response within each cell.

**GST-SNAP-23 enhances insulin-stimulated glucose uptake**

One limitation of the quantitation of GLUT4 levels in membrane lawns is that it may not distinguish between GLUT4 that is functionally incorporated into the plasma membrane from that which is merely closely associated with it (or incompletely incorporated into the membrane).
In order to determine if the changes in insulin-stimulated GLUT4 translocation observed using GST-SNAP-23 actually resulted in increased GLUT4 exposure at the cell surface we measured glucose uptake in permeabilized cells exposed to GST or GST-SNAP-23. As shown previously by us and others, limited chemical permeabilization of 3T3-L1 adipocytes with bacterial toxins still allows for measurement of 2-deoxyglucose uptake (128, 336, 375). Under these conditions, a reduced but reproducible insulin-dependent stimulation of glucose uptake is observed, allowing for the introduction of macromolecules to study their effect on insulin action. GST-SNAP-23 was introduced into SLO-permeabilized 3T3-L1 adipocytes for 15 min prior to addition of insulin for another 15 min, before glucose uptake was measured as described in Experimental Procedures. Table C3-1 shows that introduction of the fusion protein caused a significant enhancement of insulin-stimulated glucose uptake relative to cells exposed to GST or buffer alone. This supports the results in Figure 6-2 showing that microinjection of GST-SNAP-23 increases the level of GLUT4 on membrane lawns. In addition, it demonstrates that this GLUT4 is functionally incorporated into the plasma membrane lawns.
Table 6-1 Full length SNAP-23 protein improves insulin-dependent glucose uptake in SLO-permeabilized 3T3-L1 adipocytes

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative glucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>None</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>GST-SNAP-23</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>GST</td>
<td>1.02 ± 0.17</td>
</tr>
</tbody>
</table>

Monolayers of 3T3-L1 adipocytes grown in 12-well dishes were serum-starved for 2 h prior to permeabilization as described in Experimental Procedures. Permeabilization solution was replaced with solutions containing no protein, 20 μM GST-SNAP-23 or 20 μM GST and incubated for 15 min at 37°C. Half the wells were then treated with 100 nM insulin for 15 min and glucose uptake was measured as described in Experimental Procedures. Transport values are expressed as change relative to untreated controls and represent the mean ± S.E. of three independent experiments. Values significantly different from the other values in the column (p<0.05, ANOVA) are indicated by an asterisk (*).

A peptide of SNAP-23 does not affect GLUT4 translocation

Introduction of a carboxy-terminal peptide of SNAP-25 was shown to abrogate neurotransmitter release in PC12 cells (82) and in Aplysia synapses (9). In Chapter 5 we demonstrated that recombinant SNAP-23 binds directly and tightly to both syntaxin-4 and VAMP-2 in vitro. Therefore, we tested whether a peptide corresponding to the C-terminal 20 amino acids of SNAP-23 (termed SN23.c20) interfered with binding of SNAP-23 to syntaxin-4. In an in vitro binding assay a 100 times molar excess of SN23.c20 over SNAP-23 abolished SNAP-23 binding to syntaxin-4 (Figure 6-3a). No effect was seen when using an unrelated peptide instead of SN23.c20. To assess its effect on insulin-stimulated GLUT4 translocation, SN23.c20 was microinjected into 3T3-L1 adipocytes and arrival of GLUT4 at the plasma membrane was detected by indirect immunofluorescence using the plasma membrane lawn assay.
Digital image quantitation of several lawns showed that SN23.c20 did not have any significant effect on GLUT4 translocation (2.9 fold increase in GLUT4 with insulin versus 2.8 in non-injected control cells). This negative result could arise if SNAP-23 does not participate in the fusion of the GLUT4 organelle with the plasma membrane, but also if the peptide did not achieve dissociation of the binding of the endogenous SNAP-23 to syntaxin-4. It is likely that the majority of SNAP-23 in the cell is bound to syntaxin-4 because immunoprecipitation of endogenous SNAP-23 from 3T3-L1 adipocyte lysates contain syntaxin-4 (see Chapter 5).

**Figure 6-3: Microinjection of a SNAP-23 C-terminal peptide interferes with SNAP-23 binding to syntaxin 4 but does not reduce GLUT4 incorporation into the plasma membrane**

(a) The efficacy of SN23Pep in disrupting SNAP-23 binding to possible endogenous partners was assayed by an in vitro binding assay described in Experimental Procedures. 200 μM (the approximate final concentration in injected cells) SN23Pep (SN23) or UnrelPep (Unrel) were added to the SNAP-23/GST-syntaxin 4 binding assay. The proteins bound to glutathione-agarose beads after 2 h at 4°C were resolved by SDS-PAGE and GST-syntaxin 4 was detected by Ponceau S staining while SNAP-23 was immunodetected. (b) Approximately one tenth of the cell volume of 2 mM SN23Pep or UnrelPep were microinjected into ~90% of all cells in an outlined region of the coverslip. The coverslips were then serum-starved for 2 hours and incubated a further 15 min in the absence (top panels) or presence (bottom panels) of insulin. Plasma membrane lawns were prepared as described and stained for GLUT4. Indirect immunofluorescence images of GLUT4 on plasma membrane lawns from non-injected (left panels) and injected (right panels) cells were collected. Representative images from one of two experiments are shown.
Discussion

The SNARE hypothesis of synaptic vesicle exocytosis initially suggested that SNAP-25 is a t-SNARE required for docking/fusion of incoming vesicles. Along with VAMP-2 and syntaxin-1, they constitute the minimum number of proteins that can lead to membrane fusion (384). In yeast, homologues of VAMP and syntaxin are required for vacuolar fusion (239), and at least two proteins with domains resembling SNAP-25 have been described to participate in this function (239, 356). Subsets of mammalian and yeast SNAREs form very stable ternary complexes via coiled-coil interactions and the energy released from this tight binding is thought to drive membrane fusion (79, 117, 199, 384). In contrast to VAMP-2 and syntaxin-1, which are transmembrane proteins, SNAP-25 does not penetrate the membrane but associates with it through palmitoyl moieties attached to a set of four cysteine residues in the middle of the molecule (366), and by a secondary mechanism, involving coiled-coil interactions with syntaxin-1.

SNAP-25 is expressed only in neuronal and neuroendocrine cells. However, regulated membrane fusion events occur at the surface of many cells, notably exocrine secretion from vesicles and granules, and regulated exocytosis of membrane proteins such as glucose transporters in muscle and fat cells, water channels in kidney cells and proton pumps in gastric parietal cells. Rodent SNAP-23 has 57.8% identity with rodent SNAP-25 (378). Both the human and rodent SNAP-23 lack the sites required for proteolysis by Bo/NT E or A (273, 284). SNAP-23 is expressed in a variety of secretory cells, and is also present in muscle and fat cells (389). In the latter cells, its distribution is largely restricted to the plasma membrane.

The function of SNAP-23 has been tested in many systems not involving GLUT4 traffic. In one, transient expression of this protein in pancreatic islet beta cells rescued the loss of insulin secretion caused by proteolysis of the endogenous SNAP-25 with Bo/NT E (284). This
indicates that exogenous SNAP-23 has the ability to participate in membrane traffic events. In another study, proteolysis of the canine SNAP-23 -- a species isoform which is sensitive to Bo/NT E -- prevented transferrin recycling in MDCK cells (196). More recently a role a SNAP-23 was established in dense core granule release from platelets using antibodies and peptides to disturb the function of SNAP-23 (48). However, differences in the behavior of SNAP-25 and SNAP-23 have been noted in their interaction with other SNAREs in vitro. Thus, whereas SNAP-25 potentiates binding of VAMP-2 to syntaxin-1 (260), SNAP-23 appeared to decrease the association of VAMP-2 to syntaxin-4 (see Chapter 5). Moreover, the SDS-resistance displayed by in vitro complexes of recombinant proteins encoding the soluble segments of syntaxin-1, VAMP-2 and SNAP-25 was not reproduced by the equivalent segments of syntaxin-4, VAMP-2 and SNAP-23 (see Chapter 5 and (395)). This indicates that the stability of the two tripartite complexes differs. On the other hand, a possible role of SNAP-23 as a partner of syntaxin-4 and VAMP-2 in non-neuronal cells is supported the demonstration that all three co-precipitate from 3T3-L1 adipocyte cell lysates (see Chapter 5) and from αSNAP- and NSF-enriched lysates of rat fat cells (320).

A similar observation to the results presented here was recently made by Rea et al. (275) using antibodies directed to the N-terminus domain of SNAP-23. The fact that both the C-terminus and the N-terminus of SNAP-23 appear to be involved in the incorporation of GLUT4 into the membrane is, by analogy, consistent with recent reports that both the N-terminus and the C-terminus of SNAP-25 are part of the interacting core of the SNARE complex (332).

As further evidence of a functional role of SNAP-23 in insulin-stimulated GLUT4 translocation, microinjection of full length SNAP-23 into 3T3-L1 adipocytes enhanced the amount of GLUT4 present at the cell surface upon an insulin challenge (Figure 6-2). Microinjected recombinant SNAP-23, in the presence of insulin, also led to an increase in the
number of cells responding to this challenge. A change in the number of insulin-responding cells has been shown to occur in response to activated phosphatidylinositol 3'-kinase (211). Similarly, introduction of this protein by chemical permeabilization of the cells allowed for a higher insulin-dependent stimulation of glucose uptake (Table C3-I). This strongly suggests that the exogenous SNAP-23 promotes the functional incorporation of GLUT4 proteins into the membrane, presumably by facilitating fusion of the GLUT4-containing organelle with the cell surface lipid bilayer. The results presented in Figure 6-3 appear to contradict the conclusions reached from the other data presented in this Chapter since the peptide comprising the carboxy-terminal 20 amino acids of SNAP-23 was able to abolish SNAP-23 binding to syntaxin 4 in vitro but had no detectable effect on GLUT4 translocation when injected into 3T3-L1 adipocytes. The reasons for this discrepancy are unknown but could include: that the peptide is not able to reach the plasma membrane after injection due to degradation or that it is able to reach the plasma membrane but is not able to disrupt pre-existing SNAP-23/syntaxin 4 complexes.

The ability of exogenous SNAP-23 to enhance the effect of insulin is in contrast to the inhibitory effects of the cytoplasmic domains of syntaxin-4 or VAMP-2 (47, 207, 248), which presumably act as competitive inhibitors of the endogenous, membrane-bound syntaxin-4 and VAMP-2. In those studies, both molecules were missing their transmembrane domains, suggesting that this link with the appropriate membranes is critical for their function in incorporating GLUT4 compartments into the plasma membrane. In contrast, full length SNAP-23 is thought to be membrane associated via cysteine palmitoylation and protein-protein interactions, by analogy to SNAP-25 (371). We hypothesize that the microinjected SNAP-23 protein assists in incorporating the GLUT4 organelle into the plasma membrane by binding to its natural partners syntaxin-4 and VAMP-2. The exogenous SNAP-23 would act additively to the limiting amount of endogenous SNAP-23.
Recently Gould's group has performed a very thorough analysis of the quantities of SNAP-23, syntaxin 4 and VAMP-2 in 3T3-L1 adipocytes (131). Their results indicated that SNAP-23 is the most abundant of the three SNAREs in 3T3-L1 cells (11.5 x 10^5 copies/cell) followed by VAMP-2 (4.52 x 10^5 copies/cell) and then syntaxin 4 (3.74 x 10^5 copies/cell). However, the results presented in this chapter are consistent with the interpretation that SNAP-23 is in limiting amounts for the fusion of GLUT4 vesicles, so that increasing the cellular content of SNAP-23 facilitates this process. These two conclusions are not mutually exclusive since it is not clear from the quantitation (131) if all the SNAP-23 is available for binding to syntaxin 4 and/or VAMP-2. It is conceivable that SNAP-23 also functions in other fusion events throughout the cell or that its availability for fusion is regulated by other proteins such as Hrs-2 (see Chapter 5 and Discussion).

Collectively, the results presented here suggest that SNAP-23 may be a target of insulin-dependent regulation of vesicle docking/fusion in 3T3-L1 adipocytes. In addition to this work, studies from other laboratories have since confirmed that SNAP-23 is a participant in insulin-stimulated GLUT4 translocation (86, 169, 275). Including the C-terminal-directed antibodies and full-length protein used here, neutralizing antibodies as well as other peptides encoding the N- or C-termini of SNAP-23 have been introduced into 3T3-L1 adipocytes by microinjection, chemical permeabilization and adenoviral transfection. All these reagents only partially reduced the insulin-dependent arrival of GLUT4 at the plasma membrane (86, 90, 169, 275) (see Conclusions for a discussion of the possible reasons for this). These results suggest that SNAP-23 may be naturally present in limiting amounts and it may contribute with regions analogous to those of SNAP-25, in its interaction with other proteins leading to GLUT4 vesicle docking/fusion in response to insulin.
Chapter 7 – Role of VAMP-binding proteins in insulin-dependent GLUT4 translocation

Summary

SNARE proteins are required for membrane fusion, in both regulated and constitutive vesicular traffic. Because of their central role in membrane fusion, SNAREs are attractive targets for regulation, possibly by indirect regulation via other proteins that interact with them. The v-SNARE VAMP-2 is required for productive GLUT4 incorporation into the plasma membrane. VAP-33 (VAMP-associated protein of 33 kDa) and pantophysin are integral membrane proteins that bind VAMP-2 both in vitro and in vivo and thus may regulate its function. VAP-33 has a single membrane-spanning domain that is required to bind to VAMP-2 while pantophysin has four transmembrane-domains. Microinjection of VAP-33 antibodies into Aplysia californica neurons inhibited neurotransmitter secretion (309), suggesting that it participates in exocytic vesicle traffic. Pantophysin is a ubiquitous homologue of the synaptic vesicle protein synaptophysin, a protein that may regulate lipid dynamics in synaptic vesicles through its ability to bind cholesterol. Pantophysin is found on intracellular GLUT4 compartments from 3T3-L1 adipocytes and moves from an internal source to the plasma membrane in response to insulin (32). Prior to this work it was not know if VAP-33 or pantophysin participate in insulin-regulated GLUT4 traffic so the purpose of this study was to determine if either protein functions in this process. In this study we explored the subcellular distribution of VAP-33 in L6 skeletal muscle cells and 3T3-L1 adipocytes in relation to VAMP-2 and GLUT4. Furthermore we tested the participation of both VAP-33 and pantophysin in insulin-stimulated GLUT4 translocation to the plasma membrane by overexpression of wild-type VAP-33 (in L6 cells) and microinjection of antibodies and peptides (in 3T3-L1 adipocytes).

**Experimental procedures**

**Antibodies, Peptides and Western blotting.**

Affinity-purified rabbit polyclonal antibodies against a carboxy-terminal peptide of GLUT4 (372), the cytosolic domain of VAMP-2 (374), and the cytosolic domain of human VAP-33A (referred to as αVAP-33) (386) were generated as described elsewhere. At least two VAP-33 genes exist (VAP-33A and B) and the antibodies to VAP-33A recognize both VAP-33A and VAP-33B isoforms (189, 242, 317, 386). In addition, VAP-33A and VAP-33B heterodimerize (242), suggesting that they may, in fact, occur in the same subcellular locations. To avoid confusion, the term VAP-33 is used throughout this study. Rabbit polyclonal antibodies against the GST-fused carboxy-terminal 26 amino acids of murine pantophysin (referred to as αPanto) were a generous gift of Dr. Bentley Cheatham (32). Monoclonal antibodies to VAMP-2 in the form of lyophilized mouse ascites fluid (clone #69.1) were purchased from Synaptic Systems GmbH (Göttingen, Germany). Mouse monoclonal antibodies to the c-myc epitope (9E10) were obtained from two sources, Santa Cruz Biotechnology, Inc, (Santa Cruz, USA) and as a generous gift from Dr. Mike Moran (University of Toronto, Toronto, Canada), while mouse antibodies to ERGIC-53 (294) and giantin (201) were generous gifts of Dr. Hans-Peter Hauri (University of Basel, Basel, Switzerland). Secondary goat anti-rabbit conjugated HRP was obtained from BioRad (Mississauga, Canada), FITC and Cy3-labelled goat anti-rabbit and goat anti-mouse from Bio/Can Scientific (Mississauga, Canada).

Immunoblotting was performed as described elsewhere (90) with a minor adjustment in the blocking step. For immunoblots with VAP-33 antibodies, membranes were blocked with 5% milk in PBS/0.2% Tween-20 for 3 or more hours prior to incubation with primary antibody (diluted 1:250 in 5% milk/PBS/0.2% Tween-20). A GST-fused peptide comprising the carboxy-
terminal 26 amino acids of murine pantophysin (termed GST-Panto) was a generous gift of Dr. Bentley Cheatham (32).

**DNA constructs.**

Human VAP-33 cDNA encoding the full open reading frame and ~600 bp of 3’ untranslated region in pGEX-KG (Pharmacia, Baie d’Urfe, Canada) was generated elsewhere (386). A BamHI/XhoI section from this plasmid was then subcloned into pcDNA3.1+\(^\text{17}\). For expression of GFP alone the pEGFP vector from Clontech was used (Palo Alto, USA). Purified plasmid DNA of all constructs was prepared using the proprietary Maxi- or Midi-prep techniques from Qiagen (Mississauga, Canada) according to the manufacturer’s directions. VAMP-2-GFP, kindly provided by Dr. Xiao-Rong Peng, was generated by subcloning the full open reading frame of rat VAMP-2 into pEGFP. GFP-KDEL was a generous gift of Dr. Alan Verkman (University of California at San Francisco, San Francisco, USA).

**Tissue culture.**

A subclone of the L6 rat skeletal muscle cell line stably expressing GLUT4 with an exofacial myc epitope was generated as described earlier (175, 355, 380). L6-GLUT4myc myoblasts and 3T3-L1 adipocytes were grown and differentiated according to previously published protocols (355, 374, 380). Briefly, L6-GLUT4myc myoblasts were cultured in alpha modification of Eagle’s medium (αMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic/antimycotic solution (10,000 units/mL penicillin, 10 mg/mL streptomycin). 3T3-L1 adipocytes were maintained as fibroblasts in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% (v/v) calf serum (CS) and 1% (v/v)

\(^{17}\) This subcloning step was performed by M. Lynn Weir, a post-doctoral fellow in Bill Trimble’s lab with whom I collaborated.
antibiotic/antimycotic solution. 3T3-L1 fibroblasts were induced to differentiate into adipocytes by replacing the DMEM/CS with DMEM supplemented with 10% FBS, 1% (v/v) antibiotic/antimycotic solution and 0.25 μM dexamethazone, 0.5 mM 1-methyl-3-isobutylxanthine and 10 μg/mL porcine insulin. After four days the medium was changed to DMEM/FBS with 10 μg/mL insulin for an additional two days then replaced every two days with DMEM/FBS. Adipocytes were used in experiments between 11 and 14 days after initiation of differentiation. All cell types were passaged by washing with PBS, incubating with 0.05% (w/v) trypsin to lift cells off and diluted into MEM/FBS (L6-GLUT4myc myoblasts) or DMEM/CS (3T3-L1) for replating.

Subcellular fractions from confluent monolayers of these cells were generated using the technique established previously in our laboratory (372). Briefly, cells from two 10 cm dishes/condition were incubated for 3 h in serum-free medium and treated with or without 100 nM insulin for 15 min at 37°C. Monolayers were washed three times with cold homogenization buffer (255 mM sucrose, 0.5 mM PMSF, 1 μM pepstatin A, 1 μM leupeptin, 10 μM E-64, 1 mM EDTA, and 20 mM Na-HEPES; pH 7.4), scraped using a rubber policeman into 4 mL of the same buffer and homogenized by passing 20 times through a cell cracker. Fractionation of this homogenate was performed as described by Piper et al. (262), the first step of which was centrifugation at 19,000 g for 20 min to yield pellet 1 (P1) and supernatant 1 (S1). P1 was floated on a 1.12 M sucrose cushion and centrifuged for 60 min at 100,000 g. The interface layer was collected at pelleted at 50,000 for 20 min to yield partially pure plasma membrane (PM). S1 was centrifuged at 41,000 for 20 min to remove high-density microsomes and the resulting supernatant was centrifuged again at 195,000 g for 75 min to pellet the low-density microsomes (LDM). Both PM and LDM were resuspended in homogenization buffer and protein concentrations were determined by the Bradford procedure (Bio-Rad).
L6-GLUT4myc myoblasts were transiently transfected using the liposome-mediated Effectene reagent from Qiagen (Mississauga, Canada) according to the manufacturer's protocol. Briefly, 10^5 cells/well of a 6-well dish were seeded on sterile, 25 mm coverslips the day prior to transfection. On the day of transfection, plasmid DNA was first condensed using the Enhancer reagent, then packaged into liposomes consisting mainly of the proprietary reagent Effectene. These DNA-containing liposomes were then added to the cells for between 5 and 6 hours. The transfection mixture was then replaced with growth medium (10% fetal bovine serum (v/v) in α-MEM) and the cells incubated for a further 38 to 42 hours prior to use in the GLUT4-myc translocation assay (see below) or in standard immunocytochemistry (see below). This protocol consistently resulted in a transfection efficiency of between 10% and 20%. Wild-type VAP-33 was overexpressed by approximately five-fold in these cells. Transfected cells were identified by the presence of the marker constructs VAMP-2-GFP or GFP. In order to ensure that cells taking up marker constructs also took up the other constructs of interest, 0.2 μg of marker DNA was transfected per well along with 1.0 μg DNA/well of other constructs. Total DNA in each transfection was equalized to 1.2 μg/well with empty (no insert) pcDNA3.1 where needed. For experiments involving brefeldin A, cells were serum starved for 4 hours prior to a 30' treatment with brefeldin A at a final concentration of 10 μg/mL.

**Immunoprecipitation**

Whole cell lysates of 3T3-L1 mouse adipocytes were prepared as described in *Chapter 5* and pre-cleared by incubation for 2 h at 4°C with 40 μl of Protein A-Sepharose. Antibodies to VAMP-2 (2 μg IgG) were pre-coupled to Protein A-Sepharose by incubation for 2 h at 4°C in immunoprecipitation buffer (20 mM HEPES pH 7.4, 100 mM KCl, 2% Triton X-100, 2 mM EDTA). The pre-coupled beads were pelleted, washed twice with immunoprecipitation buffer
and added to the supernatant from the pre-clearing step. Lysate and antibody were incubated for 2 h at 4°C with constant rotation, and then the Sepharose was pelleted and washed three times with immunoprecipitation buffer. Fifty μl of SDS-sample buffer were added, the samples were analyzed by SDS-PAGE in 12% polyacrylamide gels, proteins were transferred to nitrocellulose and immunoblotted as described below.

**Immunooisolation of VAMP-2-containing compartments.**

The presence of VAP-33 in VAMP-2-containing compartments was detected by immunopurifying VAMP-2-containing vesicles and immunoblotting them for VAP-33. Briefly, differentiated 3T3-L1 adipocytes or L6-GLUT4myc myotubes were scraped into homogenization buffer (255 mM sucrose, 2 mM EDTA, 20 mM HEPES (pH 7.4), 200 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin A). Cells were then homogenized by 20 strokes in a glass homogenizer and the homogenate was clarified by a 5 min centrifugation at 1000 x g after which the protein concentration was measured by the BCA assay (BioRad). PBS was used to dilute 500 μg total protein of this homogenate to 900 μL. To this was added 100 μL of 1.0 M Na₂HPO₄ (pH 7.4) and 3 x 10⁷ Dynabeads (Dynal AS, Oslo, Norway) precoupled (according to manufacturer’s instructions) to 7.5 μg goat anti-rabbit IgG. Anti-VAMP-2 antibody (15 μL) or non-immune IgG (2 μg) were added to the bead/homogenate mixture and allowed to incubate overnight at 4°C with rotation. Dynabeads were collected the following day and the attached proteins eluted with SDS sample buffer containing 20 mM N-ethylmaleimide to prevent dissociation of the heavy and light chains of the immunoglobulin proteins. The proteins were then resolved by SDS-PAGE and immunoblotted for VAMP-2 and VAP-33.
**Single cell GLUT4myc translocation assay.**

The presence of GLUT4myc at the cell surface of L6 myoblasts after various treatments was measured as described previously (380). Briefly, cells transfected two days prior to performing the assay were serum-starved for 4 h at 37°C. Cells were then treated with 100 nM insulin for 20 min at 37°C and then washed 3 times with ice-cold PBS prior to a short, 2 min fixation with 4% formaldehyde in PBS (v/v). The purpose of the short fixation was to freeze the GLUT4 on the plasma membrane without disrupting plasma membrane integrity. After fixation cells were washed twice with PBS and any excess formaldehyde was reacted with 50 mM ammonium chloride for 5 min at 4°C. Following this, cells were blocked for 15 min with 5% goat serum in PBS (v/v) and then externalized myc epitope was labelled with 9E10 antibody (1 µg/mL in 5% goat serum/PBS) for 1 h at 4°C. After washing away excess antibody, the 9E10 antibody remaining on the cells was labelled with a goat anti-mouse-Cy3 conjugate in 5% goat serum/PBS for 1 h at 4°C. Again excess antibody was washed away and the cells were fixed further in 4% formaldehyde in PBS (v/v) for 30 min followed by a 10 min incubation with 50 mM ammonium chloride to react excess formaldehyde. Coverslips holding the cells were then mounted on glass slides using the AntiFade mounting solution from Molecular Probes (Eugene, USA).

Fluorescent images of labelled cells were obtained using a Leica DM-IRB inverted fluorescent microscope and Princeton Instruments (Trenton, USA) CCD camera (model RTE/CCD-1317-K/2). The NIH Image software package (NIH, Bethesda, USA) was used to digitally quantitate indirect fluorescent images of myc labelling. Fluorescent intensities from myc labelling of transfected (defined as cells expressing the marker GFP constructs) and non-transfected cells from the same field were compared to obtain a measure of the effect of the particular construct transfected. The level of myc labelling in unstimulated and insulin-
stimulated cells was also measured. Statistical analysis was performed using the Prism software package (GraphPad Software, San Diego, USA).

**Immunocytochemistry.**

After 4 h serum starvation, cells were fixed for 30 min in 4% formaldehyde in PBS at 4°C followed by a 10 min incubation in 50 mM ammonium chloride in PBS at 20°C to react excess formaldehyde. Cell membranes were then permeabilized by incubation in 0.1% Triton X-100 (v/v) in PBS for 20 min at 20°C and blocked for 30 min at 20°C with 3% milk powder and 3% goat serum (GibcoBRL) in PBS (w/v). For immunocytochemical experiments involving the monoclonal VAMP-2 antibody, a combination of 3% milk powder (w/v), 3% goat serum (v/v, GibcoBRL), 0.5% porcine skin gelatin (w/v, BioRad) and 0.2% teleostean gelatin (v/v, Sigma) in PBS was found to be required for effecting blocking of non-specific staining. Permeabilized cells were incubated with primary antibody for 1 h at 20°C, washed extensively, then incubated with secondary antibodies for 1 h at 20°C and washed again. Coverslips were then mounted and fixed onto glass slides using Dako mounting reagent (Dako Corporation, Mississauga, Canada).

Primary antibody dilutions used were the following: giantin – 1:750, αVAP-33 – 1:33, VAMP-2 – 1:500, EEA1 – 1:250, myc (9E10) – 1:200, ERGIC-53 – 1:100. Images of the resulting fluorescence patterns were collected (chosen at the plane with the highest signal intensity) using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. FITC and Cy3 conjugated secondary antibodies were used at a 1:250 dilution of the manufacturer’s recommended storage concentration. Overlaying two individual staining patterns from the same field of cells in Adobe Photoshop and selecting only the yellow colour generated images used to show colocalization of two proteins.
Microinjection/lawn assay.

Measurement of insulin-stimulated GLUT4 translocation on plasma membrane lawns was performed as described in Chapter 6. Affinity-purified αVAP-33 was dialyzed into an isotonic potassium buffer (138 mM glutamic acid; 20 mM HEPES, pH 7.2; 8 mM MgCl₂; 0.285 mM CaCl₂; 1 mM EGTA) prior to microinjection. Panto, αPanto, αVAP-33, GST or irrelevant rabbit immunoglobulins (also in the isotonic potassium buffer) were microinjected into most (>90%) of the 3T3-L1 adipocytes inside a small (1 mm²), marked area on a coverslip holding a confluent monolayer of cells. After microinjection, cells were serum starved for 90 min and either stimulated with insulin (100 nM for 15 min) or left unstimulated. GLUT4 remaining in the lawns was then detected using a primary, polyclonal GLUT4 antibody (1:150 dilution) followed by a secondary anti-rabbit-Cy3 conjugate (1:250 dilution). The primary and secondary antibodies were both diluted in 5% goat serum/PBS and incubated consecutively with the plasma membrane lawns for 1 h at 20°C. Coverslips were mounted on glass slides using the Dako mounting reagent (Dako Corporation, Mississauga, Canada). Fluorescence was visualized using a Leica DM-IRB inverted fluorescent microscope and images captured using a Princeton Instruments (Trenton, USA) CCD camera (model RTE/CCD-1317-K/2). Lawns from injected/non injected and unstimulated/insulin-stimulated cells were digitally quantitated using NIH Image software (NIH, Bethesda, USA).
Results

Figure 7-1 VAP-33 colocalized with VAMP-2

Fluorescent images of the same field of untransfected L6-GLUT4myc cells stained with antibodies to VAP-33 (centre) and VAMP-2 (left) were collected using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. The areas of overlap of the two images (right) was produced by selecting only the yellow generated by overlaying the VAP-33 and VAMP-2 images in the red and green channels of a RGB image in Photoshop. Hundreds of cells in three experiments were observed to arrive at the representative images of each staining pattern shown. The scale bar represents 20 μm.

VAMP-33 colocalizes with VAMP-2

The subcellular localization of VAP-33 was investigated by indirect confocal immunofluorescence in L6 myoblasts expressing myc-tagged GLUT4 (L6-GLUT4myc). Affinity-purified rabbit polyclonal antibodies to VAP-33 were used along with monoclonal antibodies to VAMP-2 in order to study the extent of colocalization of the two proteins. Both VAMP-2 and VAP-33 exhibited a punctate, peripheral staining throughout the cytoplasm with a slight concentration in the perinuclear region (Figure 7-1). The areas of colocalization of VAP-33 and VAMP-2 in the same field are shown in the right hand panel (only regions of colocalization are shown). The two proteins colocalized abundantly in punctate structures throughout the cytoplasm (Figure 7-1).

To complement the immunofluorescence analysis we studied the association of VAP-33 and VAMP-2 in cell lysates. We were unable to detect any VAP-33 in immune complexes of VAMP-2 from detergent solubilized lysates of 3T3-L1 adipocytes (Figure 7-2a). As a second
approach we used anti-VAMP-2 antibody to selectively sediment all VAMP-2-containing membranes out of cell homogenates and probed these for VAP-33. Figure 7-2b shows that the antibody indeed purified VAMP-2-containing bodies from both L6 myotubes and 3T3-L1 adipocytes. Importantly, VAP-33 had a marked presence in these compartments. Control experiments using non-immune IgG failed to bring down any VAMP-2 or any significant amounts of VAP-33. These results suggest that at least a fraction of the VAMP-2 and VAP-33 complements reside within the same organelle(s).

**Figure 7-2 VAP-33 is found in immunopurified VAMP-2 compartments**

Cell homogenates were prepared from L6 myotubes and 3T3-L1 adipocytes as described in *Experimental Procedures*. (a) Anti-VAMP-2 antibody or irrelevant IgG, coupled to protein A-Sepharose, was used to immunoprecipitate VAMP-2 from detergent solubilized 3T3-L1 adipocytes. (b) Anti-VAMP-2 antibody or irrelevant IgG, coupled to Dynabeads, was used to immunopurify membrane compartments containing VAMP-2 from 3T3-L1 adipocytes (lanes 1 & 3) and L6 myotubes (lane 2). The immune pellets were probed for the presence of VAMP-2 and VAP-33 by SDS-PAGE and immunoblotting with specific antibodies (see *Experimental Procedures*).
VAP-33 colocalizes with GLUT4

Figure 7-3 VAP-33 colocalized with GLUT4

Fluorescent images of the same field of L6 GLUT4myc cells stained αVAP-33 (centre) and myc (left) were collected using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. The areas of overlap of the two images (right) were produced by selecting only the yellow colour generated by overlaying the VAP-33 and GLUT4 images in the red and green channels of a RGB image in Photoshop. Hundreds of cells in three experiments were observed to arrive at the representative images of each staining pattern shown. The scale bar represents 20 μm.

To begin to explore a possible role of VAP-33 in GLUT4 traffic we determined the degree of overlap between these two proteins by immunofluorescence and subcellular fractionation. L6 GLUT4myc cells were double-labelled for the myc epitope (to detect GLUT4) and for VAP-33 (Figure 7-3). In L6 myoblasts, GLUT4myc lodges in an intracellular compartment largely devoid of GLUT1 but containing most of IRAP (355). The latter is a marker of the insulin-regulated compartment of GLUT4 in fat cells and muscle (6, 161, 170). Labelling by the anti-myc antibody presented a highly concentrated staining pattern in the perinuclear area with less intense but substantial staining dispersed throughout the cytoplasm (Figure 7-3). VAP-33 showed its typical abundant peripheral punctate pattern. GLUT4myc clearly overlapped with VAP-33 in a perinuclear region that extended somewhat into the cytoplasm, although the rest of the VAP-33 signal in the cytoplasm did not overlap with the cytoplasmic GLUT4myc. This observation suggests that only the more perinuclear bodies...
populated by VAP-33 contain GLUT4 and, based on the results in Figure 7-1, also harbour VAMP-2. Consistent with this scenario, previous studies have shown partial biochemical colocalization of GLUT4 and VAMP-2 in skeletal muscle, 3T3-L1 adipocytes and L6 muscle cells (177, 222, 271, 372).

We further explored the relationship between VAP-33 and GLUT4 by subcellular fractionation from two insulin-sensitive cells. Membrane fractions of L6 myotubes and 3T3-L1 adipocytes, typically used to analyze GLUT4 localization and translocation (262, 352), were prepared and immunoblotted for GLUT4 and VAP-33 (Figure 7-4). In both cell types, GLUT4 was more concentrated in the low-density microsomal fraction than in the plasma membrane-enriched fractions (262, 352). As expected, insulin stimulation of intact myotubes or adipocytes resulted in a substantial increase in GLUT4 recovered with the plasma membrane fraction and a concomitant decrease in GLUT4 recovered with the low density microsomal fraction, in both cell types (Figure 7-4). In contrast to GLUT4, VAP-33 was somewhat more abundant in the plasma membrane-enriched fraction than in the low-density microsomal fraction in both L6 myotubes and 3T3-L1 adipocytes, and this distribution did not change with insulin stimulation. This behavior contrasts with that of VAMP-2, which shows a modest but detectable translocation to the plasma membrane, as others have reported previously (211, 374). On SDS-PAGE, VAP-33 presented as two bands corresponding to 30 and 60 kDa (Figure 7-4), attributed to the monomer and dimer, since the 60 kDa band collapsed into the 30 kDa band in samples boiled in solution containing 5% β-mercaptoethanol (results not shown). This observation is in keeping with the previously reported dimerization of VAP-33 in vitro (242). The doublet could not be detected in Figure 7-2 because of interference by the immunoprecipitating IgG.
Figure 7-4 Effects of insulin on GLUT4 and VAP-33

Subcellular membrane fractions (PM, plasma membrane; LDM, low-density microsomes) were prepared from L6 myotubes (a and b) and 3T3-L1 adipocytes (c and d) as described in Experimental Procedures. Samples were boiled in non-reducing conditions and subsequently resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Fractions were immunoblotted for GLUT4 (a and c) or VAP-33 (b and d) and the identified bands are indicated on the right. Molecular weights of standard markers are indicated on the left. The relative amounts of the VAP-33 monomer and dimer were not consistent in three independent experiments.

VAP-33 overexpression inhibited GLUT4 translocation

The exofacial myc epitope on GLUT4 in L6-GLUT4myc cells can be used to determine the translocation and functional incorporation of the protein to the myoblast surface in response to insulin (355, 380). L6 myoblasts are easily transfectable, making it possible to test the effect of diverse proteins in GLUT4 traffic. By analogy to munc18c, which may regulate the availability of syntaxin-4 for productive fusion of GLUT4 vesicles (345), it was conceivable that
VAP-33 may regulate the availability of VAMP-2 for this process. To test this possibility, full-length VAP-33 was transiently overexpressed in L6-GLUT4myc cells. GFP was cotransfected along with VAP-33 and the expressing cells were identified by GFP fluorescence. The transfection protocol used yields virtually complete coexpression of these constructs, and expression occurs in about 20% of the cells (380). Forty-eight hours after transfection, cells were serum-deprived for 3 h and exposed to insulin for 20 min as described in *Experimental Procedures*. GLUT4myc was then detected on the surface of non-permeabilized myoblasts by labelling exposed myc epitope of GLUT4myc with appropriate antibodies (380). Within every field, GFP-positive cells were compared with vicinal GFP-negative cells. Cells transfected with the constructs of interest were analyzed in parallel to cells transfected with empty pcDNA3.1 vector and GFP cDNA. For each condition, basal and insulin-stimulated cells were analyzed. In untransfected cells, insulin caused approximately a two-fold increase in cell-surface GLUT4myc above basal level (Figure 7-5, open and diagonally hatched bars in each box). Transfection of empty pcDNA3.1 vector and GFP cDNA had no effect on either basal or insulin-stimulated levels of GLUT4myc, indicating that the transfection procedure itself did not affect the translocation machinery (Figure 7-5, first box). Overexpression of VAP-33 and GFP had no discernible effect on basal cell-surface GLUT4myc levels. Importantly, cells expressing exogenous VAP-33 showed approximately 60% less GLUT4myc on their surface after insulin treatment relative to insulin-stimulated, untransfected cells or empty vector-transfected cells (p<0.01 versus all other values, Tukey’s Analysis of Variance) (Figure 7-5, second box).

Given that VAMP-2 is required for GLUT4 translocation and is able to bind VAP-33, it was plausible that the overexpressed VAP-33 might have scavenged away VAMP-2, retaining it in an immobile internal compartment and preventing it from functioning as a fusogen. To explore this possibility, VAMP-2-GFP was overexpressed in the absence or presence of VAP-33.
and the cell surface GLUT4myc levels were measured before and after insulin stimulation as described above. Overexpression of VAMP-2-GFP on its own did not affect basal levels of GLUT4myc and showed a tendency to enhance insulin-stimulated GLUT4myc translocation, although without reaching statistical significance (p>0.05, Tukey’s Analysis of Variance) (Figure 7-5, third box). Interestingly, VAMP-2-GFP co-overexpression rescued the inhibitory effect of VAP-33 (Figure 7-5, fourth box). Under these conditions, the insulin-stimulated levels of cell surface GLUT4myc were not significantly different from those in insulin-stimulated and untransfected, or in insulin-stimulated cells transfected with empty vector (p>0.05, Tukey’s Analysis of Variance). These results suggest that VAP-33 performs an important, at least facilitative role, in the process of GLUT4-vesicle fusion and productive GLUT4 incorporation into the cell surface. It is possible that VAP-33 may limit the amount of endogenous VAMP-2 available to mediate GLUT4myc vesicle fusion with the plasma membrane.
Overexpressed VAP-33 inhibited insulin-stimulated GLUT4 translocation

Insulin-stimulated GLUT4 translocation measured in LGGLUT4myc cells by the GLUT4myc translocation assay (see Experimental Procedures) was used to monitor the effect of overexpression of VAP-33 and VAMP-2 constructs. For pcDNA3.1 and VAP-33 conditions, GFP was cotransfected to allow transfected cells to be easily identified. Total DNA in each condition was equalized to 1.2 µg/well with additional pcDNA3.1 DNA as required. Digital images of cell surface GLUT4myc from untransfected and transfected, both unstimulated and insulin-stimulated, for each construct were quantitated and expressed relative to basal untransfected values of cells in the same experiment. Shown are the mean and standard error of four independent experiments. Statistical analysis was performed using Tukey's Analysis of Variance (* = measurements significantly different from relevant basal values, ** = measurement significantly different from all other values).
Perturbation of VAP-33 and pantophysin inhibited GLUT4 translocation

Figures 7-1 and 7-3 showed that VAP-33 colocalized best with VAMP-2 and to some extent with GLUT4myc, and that the VAP-33/VAMP-2 combination appeared to be important for GLUT4myc externalization in response to insulin. Previous work has demonstrated that the VAMP-2-binding protein pantophysin is found on immunopurified GLUT4 vesicles and moves to the plasma membrane in response to insulin (32), suggesting that it too may be involved in GLUT4 traffic by virtue of its proximity to that transporter. The participation of VAMP-2 and its SNARE partners syntaxin-4 and SNAP-23 in GLUT4 traffic has been well characterized in 3T3-L1 adipocytes, through the use of peptides, neutralizing antibodies or toxins microinjected into these cells prior to insulin stimulation (90, 222, 275, 375). Given the success of this experimental paradigm in testing the contribution of the SNARE proteins to GLUT4 traffic we used similar methods to disrupt VAP-33 and pantophysin function. A GST-fusion peptide of pantophysin (GST-Panto) and antibodies to VAP-33 or pantophysin were microinjected into 3T3-L1 adipocytes, under the same conditions in which we and others previously demonstrated the need for syntaxin-4 and SNAP-23 (90, 275, 340, 375). Arrival of GLUT4 at the cell surface of 3T3-L1 adipocytes at 15 min insulin stimulation was scored by immunodetection on adhered plasma membrane lawns (90, 340) as described in Experimental Procedures. Typical fields of lawns from stimulated or unstimulated cells not microinjected or microinjected with either irrelevant IgG, anti-VAP-33 antibodies (Figure 7-6), anti-pantophysin antibodies (Figure 7-7) or GST-Panto (Figure 7-8) are presented. Digital quantitation of fluorescent images of these and additional similar lawns (Figure 7-6, 7, 8) revealed that all three reagents decreased the insulin-stimulated GLUT4 translocation to a significant extent (p<0.01, ANOVA). In contrast, none of
the reagents affected the basal levels of GLUT4 detected on the plasma membrane lawns after a 90 min incubation following microinjection (Figure 7-6, 7, 8). These results suggest that the reagents interfered with the insulin-dependent GLUT4 traffic rather than with recycling of GLUT4 in the basal state.

Due to the experimental design the effects observed here represent the minimum effect of each reagent since invariably some of the lawns counted as ‘injected’ are in fact not from injected cells. This happens because only about 90% of cells in the defined ‘injected’ area actually get injected. This has the effect of decreasing any difference between the ‘injected’ and ‘non-injected’ measurements, be they positive or negative. Thus the reagents used here were at least as effective as indicated in Figures 7-6, 7 and 8.
Affinity-purified polyclonal antibodies raised against the cytoplasmic domain of VAP-33 were microinjected into 3T3-L1 adipocytes and their effects on insulin-stimulated GLUT4 translocation measured by the plasma membrane lawn assay as described in Experimental Procedures. (a) Representative GLUT-4-stained lawns from non-injected, anti-VAP-33-injected (αVAP-33), and irrelevant IgG-injected (IgG) cells pretreated with (Insulin) or without (Basal) 100 nM insulin for 15 min. (b) Fold stimulations over basal (open bars) caused by insulin (black bars) were calculated from data obtained by digital quantitation of lawns from injected and vicinal, non-injected cells. Folds were expressed relative to lawns from basal, non-injected cells prepared on the same day. Shown are the mean and standard error of four independent experiments for both αVAP-33 antibodies and irrelevant rabbit IgG. Asterisk (*) indicates result was significantly different from non-injected and IgG-injected controls (p < 0.01, ANOVA).
Affinity-purified polyclonal antibodies raised against the carboxy-terminal 26 amino acids of pantophysin were microinjected into 3T3-L1 adipocytes and their effects on insulin-stimulated GLUT4 translocation measured by the plasma membrane lawn assay as described in Experimental Procedures. (a) Representative GLUT-4-stained lawns from non-injected, αPanto-injected (αPanto), and irrelevant IgG-injected (IgG) cells pretreated with (Insulin) or without (Basal) 100 nM insulin for 15 min. (b) Fold stimulations over basal (open bars) caused by insulin (black bars) were calculated from data obtained by digital quantitation of lawns from injected and vicinal, non-injected cells. Folds were expressed relative to lawns from basal, non-injected cells prepared on the same day. Shown are the mean and standard error of four independent experiments for both αPanto antibodies and irrelevant rabbit IgG. Asterisk (*) indicates result was significantly different from non-injected and IgG-injected controls (p < 0.01, ANOVA).
Figure 7-8 GST-Panto inhibited insulin-stimulated GLUT4 translocation

A GST-fusion peptide comprising the carboxy-terminal 26 amino acids of pantophysin fused to GST was microinjected into 3T3-L1 adipocytes and its effects on insulin-stimulated GLUT4 translocation measured by the plasma membrane lawn assay as described in Experimental Procedures. (a) Representative GLUT-4-stained lawns from non-injected, GST-Panto (Panto), and GST-injected cells pretreated with (Insulin) or without (Basal) 100 nM insulin for 15 min. (b) Fold stimulations over basal (open bars) caused by insulin (black bars) were calculated from data obtained by digital quantitation of lawns from injected and vicinal, non-injected cells. Folds were expressed relative to lawns from basal, non-injected cells prepared on the same day. Shown are the mean and standard error of three independent experiments for both Panto & GST. Asterisk (*) indicates result was significantly different from non-injected and GST-injected controls (p < 0.01, ANOVA).
Figure 4-9 Presence of VAP-33 in the ERGIC and Golgi compartment

Affinity-purified rabbit polyclonal antibodies to VAP-33 (centre column) were used to label this protein in L6-GLUT4myc cells double-labelled using mouse monoclonal antibodies to (a) ERGIC-53 and (a, b) giantin (left panels as indicated). (b) Prior to preparation for immunofluorescence cells were incubated for 30 min in the absence ((top panels) or presence (bottom panels) of 5 μg/mL brefeldin A. Fluorescent images were collected using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. The areas of overlap of the two images (right column) was produced by selecting only the yellow generated by overlaying the left and central images in the red and green channels of a RGB image in Photoshop. Hundreds of cells in three experiments were observed to arrive at the representative images of each staining pattern shown. The scale bar represents 20 μm.

VAP-33 in the Golgi and the ERGIC

The above results suggest that VAP-33, VAMP-2 and GLUT4 are physically and functionally interrelated in insulin-sensitive cells. Very recent studies have analyzed the distribution of VAP-33 isoforms in epithelial and neuronal cells (189, 308). In liver, VAP-33 was found both in the apical plasma membrane and in undefined intracellular compartments
(189); in COS cells it was found in the ERGIC (Weir, M. L., Klip, A., and Trimble, W.S., unpublished), and in brain slices and cortical cells it was found in the endoplasmic reticulum and associated with microtubules (308). These results prompted us to examine whether VAP-33 may also populate any of these compartments in L6 myoblasts. As shown above, our biochemical analysis showed that a fraction of VAP-33 is recovered with isolated plasma membranes and light microsomes, and that a significant portion was associated with immunopurified VAMP-2-containing compartments. This latter result was confirmed by immunofluorescence, however a portion of VAP-33 escaped colocalization with VAMP-2. To examine the nature of this additional compartment, we used antibodies to ERGIC-53 (ER-Golgi intermediate compartment protein of 53 kDa) and giantin (a Golgi marker, proposed to be a vesicle tethering protein) (Figure 7-9a). ERGIC-53 showed a spot-like distribution throughout the cell with a considerable concentration in a perinuclear area. However, the areas of coincidence of ERGIC-53 and VAP-33 in the same field of cells were small. Giantin was observed in an extremely tight, perinuclear area, as has been described in other systems (201). In this region, VAP-33 also showed only a minor degree of overlap with giantin (Figure 7-9a). As additional evidence that VAP-33 does not significantly populate the Golgi compartment the fungal metabolite brefeldin A, which causes redistribution of the Golgi, had no perceptible effect on VAP-33 localization (Figure 7-9b). The ER was identified from the fluorescence of a transfected green fluorescent protein fusion construct containing a luminal ER-targeting sequence (GFP-KDEL). Only a minute colocalization of endogenous VAP-33 and transfected GFP-KDEL was observed (results not shown). The low amounts of VAP-33 found colocalizing with biosynthetic pathway markers may represent newly synthesized VAP-33 progressing through these compartments, although a functional role for VAP-33 in these organelles cannot be excluded.
**VAP-33 in the recycling endosome**

The disperse, punctate aspect of VAP-33 distribution suggested the possibility that it may populate one or more of the endosomal bodies. It has been shown that, in addition to their presence in granules and exocytic vesicles, VAMPs can also be present and function in the recycling endosomes (391). The strong co-localization of VAP-33 with VAMP-2 led us to examine whether the former protein could be detected in this compartment. TfR is a transmembrane protein involved in recycling of the iron scavenging protein transferrin (185, 388) and often used as a marker of the recycling endosome. Figure 7-10 depicts the staining patterns of VAP-33 and TfR. In contrast to the perinuclear plus cytoplasmic localization of VAP-33 the TfR was only perinuclear. This localization was not affected by brefeldin A treatment (results not shown), in contrast to the dispersion of giantin caused by this agent (Figure 7-9b), confirming that TfR was found in the recycling endosome and not the Golgi apparatus. In spite of the different pattern displayed by VAP-33 and TfR, the two proteins overlapped in the perinuclear area (Figure 7-10). These results suggest that the organelles rich in VAP-33 may include the recycling endosome. In experiments not shown, the disperse punctate pattern of VAP-33 was found to differ from that of EEA-1 (early endosomal antigen-1), which appeared as larger bodies through the cytoplasm.
Figure 7-10 Presence of VAP-33 in the recycling endosome.

Affinity-purified rabbit polyclonal antibodies to VAP-33 (centre panel) were used to label this protein in L6-GLUT4myc cells double-labelled using mouse monoclonal antibodies to transferrin receptor (left panel). Fluorescent images were collected using a Zeiss Axiovert 100M Laser Scanning Confocal Microscope 510. The areas of overlap of the two images (right column) was produced by selecting only the yellow generated by overlaying the left and central images in the red and green channels of a RGB image in Photoshop. Hundreds of cells in three experiments were observed to arrive at the representative images of each staining pattern shown. The scale bar represents 20 μm.

Discussion

The purpose of this study was to investigate the localization and function of the VAMP-interacting proteins VAP-33 and pantophysin in a mammalian cell with regulated vesicular traffic. The spatial relationship between pantophysin, GLUT4 and VAMP-2 has been studied previously (32), so in this study we defined the spatial relationship between VAP-33, VAMP-2 and GLUT4. Moreover, we searched for a possible role for both pantophysin and VAP-33 in GLUT4 traffic by the following strategies: overexpression of the VAP-33, microinjection of neutralizing antibodies against both proteins and microinjection of a peptide of pantophysin.

Isoforms of VAP-33

The discovery of three VAP-33 isoforms begs the question of whether they share a subcellular localization. The antibodies used to date by us and others were raised to cytoplasmic domains of VAP-33a ((189, 309) and this study), or to full-length VAP-33b (317). Regrettably, it is not yet known if any of these antibodies cross-react with isoforms other than the one they were
raised against. Using an antibody raised to VAP-33a for confocal immunofluorescence studies in numerous epithelial cell lines, the reacting protein was found to be predominantly in the plasma membrane (189). This contrasts with the ER and Golgi localization observed using an antibody raised against VAP-33b in CHO cells (317). In the present study we used an affinity-purified antibody raised to the cytoplasmic portion to VAP-33a and observed a disperse distribution that escaped a strict localization to the ER or Golgi apparatus (Figure 7-9) as well as the recycling endosome (Figure 7-10). The question of which isoform is detected by our antibody remains open. By Northern blotting approximately equal levels of mRNA of both VAP-33a and VAP-33b exist in L6 myoblasts and 3T3-L1 adipocytes (M. L. Weir & W. S. Trimble, personal communication). These two isoforms may have a similar localization, however, given that VAP-33a dimerizes in vitro with itself and with VAP-33b (242). Indeed, the dimers were observed even in SDS-PAGE (Figure 7-4), suggesting that the interaction is strong and resistant to detergent. Even though it still remains to be determined whether the two isoforms interact in vivo or, if not, which isoform is detected by the available antibodies, our results show that VAP-33 and VAMP-2 have partial colocalization in a mammalian cell, and that interfering with VAP-33 levels inhibits the insulin-dependent GLUT4 translocation to the cell surface in both L6 myoblasts and 3T3-L1 adipocytes.

**VAP-33 colocalizes with VAMP-2 and GLUT4**

By indirect confocal immunofluorescence in L6 myoblasts, VAP-33 was found to be widely distributed throughout the cell, with a slight enrichment around the nucleus. This localization was significantly, though not entirely, shared by VAMP-2. VAP-33 also colocalized in part with GLUT4 (Figure 7-3) as has been reported for VAMP-2 (271). A lesser, though detectable overlap was found between VAP-33 and Tlr, but only a minute colocalization was
found with markers of the ER, ERGIC and Golgi (Figure 7-9). By subcellular fractionation, VAP-33 (Figure 7-4) and pantophysin (32) are in intracellular membranes but also to some degree in plasma membranes. However, subcellular fractions prepared from insulin-treated cells display decreased levels of pantophysin in the intracellular fractions with corresponding increases in the plasma membrane fractions, similar to the phenomenon observed for GLUT4 but different from that observed for VAP-33. VAP-33 did not appear to change locations in either 3T3-L1 adipocytes or L6 myotubes treated with insulin. By immunofluorescence, only a fraction of VAP-33 colocalized with GLUT4. This may explain why, by subcellular fractionation, we were unable to observe any change in VAP-33 localization in response to insulin, even if both proteins were to populate the insulin-sensitive compartment. A similar observation has been made earlier for the secretory compartment associated membrane protein SCAMP (190). It is also possible that VAP-33, GLUT4 and VAMP-2 may colocalize at the level of the sorting endosome and/or the recycling endosome, where VAP-33 and VAMP-2 could potentially control GLUT4 traffic.

Immunoprecipitates of pantophysin have been shown to contain VAMP-2 (32) but we were unable to find VAMP-2 in VAP-33 immunoprecipitates (Figure 7-2a). Since VAP-33 and VAMP-2 are known to interact through their transmembrane domains (a hydrophobic interaction most likely) (386) we believe that the detergents required to solubilize the membranes for immunoprecipitation also disrupt the interaction between VAP-33 and VAMP-2. Therefore, we immunopurified VAMP-2-containing membrane bodies and found that they contain significant amounts of VAP-33 (Figure 7-2). We interpret these collective results to indicate a substantial topological coincidence of VAP-33 and VAMP-2 in these cells.
Functional role of VAP-33 and pantophysin in GLUT4 traffic

In the present study we provide evidence that VAP-33 and pantophysin are required for the productive fusion of GLUT4 vesicles with the plasma membrane. Overexpression of VAP-33 in L6 myoblasts (Figure 7-5) resulted in a sharp diminution of insulin-induced GLUT4myc externalization in L6 myoblasts (by 60%). Most interesting was the observation that overexpression of VAMP-2-GFP along with VAP-33 caused complete recovery of the inhibitory action on GLUT4myc translocation caused by VAP-33 alone (Figure 7-5). The simplest interpretation of this result is that excess VAP-33 prevents the normal function of endogenous VAMP-2, possibly by preventing it from binding to other SNARE molecules and from participating in subsequent vesicle fusion. This hypothesis implies that the ratio of VAMP-2 to VAP-33 is critical for accurate traffic of GLUT4 after insulin stimulation.

Microinjection of antibodies against- and peptides of SNARE proteins is a successful approach utilized to underscore the importance of these molecules in GLUT4 vesicle incorporation into the plasma membrane (47, 90, 275). Affinity-purified VAP-33 and pantophysin antibodies clearly attenuated insulin-stimulated GLUT4 translocation to the plasma membrane of 3T3-L1 adipocytes as measured on membrane lawns (Figure 7-6,7), as did a peptide of pantophysin (Figure 7-8). The apparently conflicting results in Figures 7-5 and 7-6 are not inconsistent with the hypothesis that an optimal ratio of VAMP-2/VAP-33 is critical for vesicle fusion. The neutralizing antibody may bind to VAMP-2/VAP-33 complexes, precluding VAMP-2 from binding to other partners. Of course, it cannot be ruled out at present that VAP-33 may also bind to other molecules essential for vesicle fusion and that the antibody interferes with these interactions. Future studies are necessary to elucidate the VAP-33 regions engaged by the antibody and those binding VAMP-2, as well as to understand how binding of VAP-33 to VAMP-2 affects SNARE complex formation. The concept that an ideal ratio must exist between
interacting proteins important for vesicle traffic is not unprecedented. Indeed, rab4 or rab4 mutants affected GLUT4 traffic differentially depending on their level of expression (61). Similarly, an optimal amount of munc-18c relative to syntaxin-4 is critical for either promoting or inhibiting GLUT4-vesicle fusion with the plasma membrane (345). For pantophysin the data shown in Figures 7-7 and 7-8 suggest a function for this protein in insulin-mediated GLUT4 traffic and, since pantophysin is found on GLUT4 vesicles, it seems likely that this protein has a more intimate role in the actual fusion process than does VAP-33.

The studies reported here and elsewhere have begun to identify proteins important for vesicle traffic, both in regulated GLUT4 externalization and in other systems. Since VAMPs are required in diverse vesicle traffic events (7, 47, 150, 153) and both VAP-33 (386) and pantophysin (32) are broadly expressed, the results presented here suggest that VAP-33 and pantophysin may regulate traffic in many systems, beyond insulin-stimulated GLUT4 translocation. More quantitative approaches, including determination of the VAMP-2/VAP-33 stoichiometric ratios and of the range of VAP-33 and pantophysin partners and their localizations will be required to determine how the many different proteins involved contribute to the final incorporation of vesicles into their target membrane.
Overall discussion & future directions

The nature of the compartments populated by GLUT4 and how GLUT4 moves through them is still poorly understood despite extensive efforts by many groups. Studies over the past five years have focused mostly on individual proteins found associated with GLUT4 compartments. Such work has made inroads into understanding the function of those proteins and the resulting knowledge has been incorporated into current models of GLUT4 traffic. However, there are many aspects of these models that have not been addressed experimentally. For instance, are the compartments that move GLUT4 to the plasma membrane preformed or does insulin induce their genesis? At how many loci does insulin act at to stimulate GLUT4 movement to the plasma membrane? Importantly, is fusion with the plasma membrane one of those regulated steps or does the fusion machinery act in an unregulated fashion and insulin’s function is to generate more vesicles to carry GLUT4? At a more molecular level, what are the macromolecules that mediate all of these functions? Chapter 4 presented results that address the question of whether insulin acts to enhance GLUT4 transit and GLUT4 vesicle genesis while Chapters 5 through 7 focused on the some of the proteins that mediate GLUT4 vesicle traffic and fusion. The Discussion sections of the individual chapters summarized how the findings fit with existing literature to develop a more comprehensive model of GLUT4 traffic. This section will focus on what approaches might by used to address some of the unresolved question in GLUT4 traffic and on some of the more speculative implications of the results presented in the data chapters. These questions include: future directions of the study of inter-endosomal traffic of GLUT4, some of the unresolved controversies in the study of SNARE protein action in GLUT4 vesicle fusion and whether SNAP-23, VAMP-2 and syntaxin 4 are the only SNAREs that mediate insulin-stimulated GLUT4 vesicle fusion with the plasma membrane.
Inter-endosomal traffic of GLUT4

For the past decade the primary acute effect of insulin on GLUT4 traffic was thought to be only on the externalization of GLUT4. For this reason most of the functional studies on GLUT4 traffic have focused on the fusion of GLUT4 vesicles with the plasma membrane. Given that GLUT4 incorporation into the cell surface appears to fail in insulin resistance accompanying several forms of diabetes (172, 176, 401, 402) the results presented in Chapter 4 indicate that intracellular traffic of GLUT4 may also be important for insulin's recruitment of GLUT4 to the plasma membrane. How the intracellular traffic of GLUT4 might be studied in greater detail is discussed below.

Different intracellular events depend on PI3K to different extents (13). On one extreme, insulin-stimulated GLUT4 movement to the plasma membrane has an absolute requirement for PI3K activity – in the absence of the enzyme's activity there is no translocation (13, 46, 56, 301, 351). On the other hand, inter-endosomal movement of Semliki Forest Virus and platelet-derived growth factor receptor exhibits only a modest slowing in the presence of wortmannin concentrations sufficient to abolish PI3K activity (13, 157, 212). In the data presented in Chapter 4 disruption of PI3K activity appeared to abolish the acceleration of inter-endosomal GLUT4 traffic stimulated by insulin. That this interference with PI3K only abrogated insulin-stimulated effects on GLUT4 traffic and did not affect unstimulated inter-endosomal traffic to an extent detectable in this assay suggests that this action of PI3K is specific to insulin action and not a general effect on endosomal traffic. These questions could potentially be resolved by the development of cell-free assays to measure the various inter-compartmental movements that GLUT4 makes, including movement through the recycling endosome and fusion with the plasma membrane. The more quantitative measurements that might be obtained from such systems would reveal just how much of an effect insulin has on inter-endosomal traffic and how that
effect is altered by interference with PI3K. The interpretation of results from experiments designed to perturb PI3K activity are complicated by the involvement of PI3K in numerous signalling pathways and traffic events (13, 362, 363). Reconstitution of GLUT4 traffic events in vitro would help to isolate the system from other confounding factors, thereby clarifying the role of proteins such as PI3K. In addition, it would permit molecular dissection of the individual steps, allowing identification of those proteins that are responding to PKB and the lipid products of PI3K.

Development of such an assay would also aid in identification of other components of the machinery responsible for inter-endosomal of GLUT4 traffic. For instance, a role for Rab4 in GLUT4 traffic has been established but the steps Rab4 regulates are not known. Since Rab4 is found on both early endosomes and recycling endosomes it could potentially modulate movement of GLUT4 between those two compartments rather than to the plasma membrane as has been suggested (62, 299). Disruption of Rab4 function in the early to recycling endosome pathway would affect downstream traffic of GLUT4 and result in decreased GLUT4 recruited to the plasma membrane in response to insulin.

While reconstitution of inter-endosomal GLUT4 traffic in vitro would be very beneficial for dissecting its components, it would require a large effort to develop. An intermediate step towards in vitro reconstitution might be the development of a cracked cell system. Such a system would allow antibodies to be introduced to block certain steps or allow cytosol depleted of certain components to be tested for its ability to mediate inter-endosomal traffic. However it is addressed, the regulation of inter-endosomal traffic of GLUT4 is now an important aspect of insulin action.


**SNAREs mediating GLUT4 externalization**

Given that SNARE proteins are required for membrane fusion, that SNAP-23 and syntaxin 4 are on the plasma membranes of insulin-sensitive tissues and that VAMP-2 is found on intracellular GLUT4 compartments, the participation of these three SNAREs in GLUT4 incorporation into the plasma membrane is not surprising. With the demonstration here (see Chapter 6) and by other groups (86, 169, 275) of the participation of SNAP-23 in insulin-stimulated GLUT4 traffic, the combination of SNAP-23, syntaxin 4 and VAMP-2 now represents a cognate set of SNARE proteins. This group of proteins likely works together to promote fusion of a portion of GLUT4 vesicles with the plasma membrane of fat and skeletal muscle cells in response to insulin.

The participation of these three proteins is supported by ample evidence in diverse systems using several different techniques, all of which are summarized in Table D-1. Two discrepancies are apparent in these results. The first is the effect of Bo/NT E on rodent SNAP-23 and the second is the effect of adding exogenous full-length SNAP-23 on insulin-stimulated GLUT4 traffic. Bo/NT E, whose primary *in vivo* target is likely SNAP-25 (292), can also cleave SNAP-23 from certain species, notably canine (196). Two experiments in 3T3-L1 adipocytes have reported an Bo/NT E-mediated inhibition of insulin-stimulated GLUT4 movement to the plasma membrane (86, 205), however, Macaulay et al (205) also demonstrated that Bo/NT E did not affect murine SNAP-23 *in vitro* suggesting that Bo/NT E might be acting through another molecule within the cell. Subsequently Foran *et al.* reported that Bo/NT E could cleave SNAP-23 in digitonin-permeabilized 3T3-L1 adipocytes (86)! The most obvious explanation for this discrepancy is that murine SNAP-23 is an inefficient substrate for Bo/NT E and that a large molar ratio of enzyme to substrate is required to cleave it (e.g. that Foran *et al.* managed to achieve and that Macaulay *et al.* did not). Due to experimental differences the results of the two
groups regarding the cleavage of SNAP-23 are not directly comparable so it seems reasonable that Foran et al. (86) saw an effect of Bo/NT E on SNAP-23 at higher enzyme to substrate ratios than those used by Macaulay et al. (205).

In Chapter 5 we demonstrated that introduction of exogenous full-length SNAP-23 into 3T3-L1 adipocytes enhanced both insulin-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. Recently, Kawanishi et al. showed that overexpression of SNAP-23 in 3T3-L1 adipocytes did not enhance insulin-stimulated GLUT4 translocation (169). Again, the reasons for this discrepancy are unclear but perhaps the most significant difference between the two experimental paradigms is the method of introducing SNAP-23. In the experiments detailed in Chapter 5 we introduced SNAP-23 by single cell microinjection (to measure GLUT4 translocation) or by SLO-mediated permeabilization (to measure glucose uptake). These two techniques introduced SNAP-23 into the cells very quickly, allowing very little time for the cell to adjust itself to compensate for possible effects of the exogenous protein. Kawanishi et al. transiently transfected SNAP-23 cDNA into 3T3-L1 adipocytes and allowed the cells to express the protein for 48 hrs prior to use in experiments (169). The extended exposure of the cells to excess SNAP-23 may allow the cells to alter levels of other proteins in order to compensate for the unnatural levels of SNAP-23.

**Do VAMP-2, SNAP-23 and syntaxin 4 suffice to cause GLUT4 vesicle fusion?**

The SNAREs syntaxin 4, VAMP-2 and SNAP-23 appear to be essential for a significant fraction of the insulin-stimulated GLUT4 arrival at the cell surface of fat and muscle cells (see Table D-I for summary). However, in all the experiments discussed above, interfering with VAMP-2, syntaxin 4 or SNAP-23 only partially inhibited insulin-stimulated GLUT4
translocation, and the basal levels of plasma membrane GLUT4 were not altered even after long time periods in the presence of the perturbing agent. This latter observation is especially surprising given that GLUT4 is known to cycle dynamically to and from the membrane in the absence of insulin. These observations suggest a number of things: 1) that SNAP-23, VAMP-2 and syntaxin 4 have no role in constitutive GLUT4 traffic, 2) that insulin may draw GLUT4 from multiple compartments and 3) that each of these compartments may require a distinct set of SNARE proteins for fusion with the plasma membrane. Of course, it is also possible that in each experiment the specific SNARE protein interfered with retained some of its functionality resulting in incomplete inhibition of GLUT4 traffic, although this seems unlikely given that similar results were obtained with diverse techniques. In addition, if insulin draws GLUT4 from two (or more) compartments then it seems possible, even likely, that the SNAP-23/syntaxin 4/VAMP-2-independent compartment is also the compartment from which GLUT4 cycles constitutively. The identity of this compartment remains elusive but a very recent morphological study suggests that it may be an endosomal/trans-Golgi network system (270). Future work in this area should direct efforts towards further characterizing this compartment, including the identification of the SNAREs required for its incorporation into the plasma membrane. Some of the SNAREs that may be involved are syntaxin 2, syntaxin 3 and VAMP-3. Other than VAMP-2, the only other v-SNARE that has been detected in the appropriate subcellular location of muscle and fat cells is VAMP-3. However, complete hydrolysis of this protein by botulinum toxins, tetanus toxins or peptides emulating the amino-terminal sequences of VAMP-3 failed to affect either basal or insulin-mediated GLUT4 arrival at the cell surface (207, 248, 271), whereas the analogous domain of VAMP-2 effectively inhibited half of the insulin action (207). It is conceivable that muscle and fat cells express other, toxin-insensitive VAMPs that could potentially mediate the fusion events that are not accounted for by VAMP-2. A likely candidate
for this function is VAMP-8, which has been implicated in another plasma membrane-directed vesicle fusion event; secretory granule exocytosis in mast cells (255). VAMP-8, or endobrevin, is not cleaved by tetanus toxin and so could potentially account for the VAMP-2-independent GLUT4 traffic. Similarly, it is conceivable that syntaxins 2 or 3 could also mediate GLUT4 vesicle fusion events since they both bind to VAMP-2 (78, 266) and are found in at least some insulin-responsive tissues.

Table D-I: Experiments demonstrating functional involvement of SNAREs

<table>
<thead>
<tr>
<th>VAMP-2</th>
<th>GLUT4 translocation</th>
<th>Glucose uptake</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Reagent and method of introduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Botulinum toxin D by Streptolysin-O toxin</td>
<td>Decreased</td>
<td>n. d.</td>
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<tr>
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<td>Botulinum toxin B by Streptolysin-O toxin</td>
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<td>Decreased</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Cytoplasmic domain by Streptolysin-O toxin</td>
<td>Decreased</td>
<td>n. d.</td>
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<tr>
<td>3T3-L1</td>
<td>N-terminal peptide by microinjection</td>
<td>Decreased</td>
<td>n. d.</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Tetanus toxin by microinjection</td>
<td>Decreased</td>
<td>n. d.</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Botulinum toxin B by microinjection</td>
<td>Decreased</td>
<td>n. d.</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Botulinum toxin B by toxin-mediated uptake</td>
<td>n. d.</td>
<td>Decreased</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Cytoplasmic domain by adenoviral transfection</td>
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<td>n. d.</td>
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<tr>
<td>Rat adipocytes</td>
<td>Tetanus toxin by electroporation</td>
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<td>No effect</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>N-terminal peptide by Streptolysin-O toxin</td>
<td>Decreased</td>
<td>n. d.</td>
</tr>
<tr>
<td>L6 myoblasts</td>
<td>Tetanus toxin by transfection</td>
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<td>n. d.</td>
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<table>
<thead>
<tr>
<th>Syntaxin 4</th>
<th>GLUT4 translocation</th>
<th>Glucose uptake</th>
<th>Ref.</th>
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<td>Cell type</td>
<td>Reagent and method of introduction</td>
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<td></td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Cytoplasmic domain by Streptolysin-O toxin</td>
<td>Decreased</td>
<td>n. d.</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Antibody by Streptolysin-O toxin</td>
<td>n. d.</td>
<td>Decreased</td>
</tr>
</tbody>
</table>
### 3T3-L1
- Internal peptide (106-22) by microinjection: Decreased, n. d. (203)
- Cytoplasmic domain by adenoviral transfection: Decreased, n. d. (248)
- Cytoplasmic domain by microinjection: Decreased, n. d. (248)
- Antibody by microinjection: Decreased, n. d. (340)

### SNAP-23

<table>
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<tr>
<th>Cell type</th>
<th>Reagent and method of introduction</th>
<th>GLUT4 translocation</th>
<th>Glucose uptake</th>
<th>Ref.</th>
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<td>n. d.</td>
<td>(205)</td>
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<td>n. d.</td>
<td>(275)</td>
</tr>
<tr>
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<td>N-terminal antibody by microinjection</td>
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<td>n. d.</td>
<td>(275)</td>
</tr>
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<td>3T3-L1</td>
<td>C-terminal antibody by microinjection</td>
<td>Decreased</td>
<td>n. d.</td>
<td>(90), Ch. 6</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Full-length protein by microinjection</td>
<td>Increased</td>
<td>n. d.</td>
<td>(90), Ch. 6</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>Full-length protein by Streptolysin-O toxin</td>
<td>n. d.</td>
<td>Increased</td>
<td>(90), Ch. 6</td>
</tr>
<tr>
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<td>n. d.</td>
<td>(86)</td>
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<td>No effect</td>
<td>(169)</td>
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<tr>
<td>3T3-L1</td>
<td>N-terminal 202 amino acids by adenoviral transfection</td>
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<td>Decreased</td>
<td>(169)</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>C-terminal 20 amino acids by microinjection</td>
<td>No effect</td>
<td>n.d.</td>
<td>Ch. 3</td>
</tr>
<tr>
<td>3T3-L1</td>
<td>N-terminal 161 amino acids by adenoviral transfection</td>
<td>No effect</td>
<td>No effect</td>
<td>(169)</td>
</tr>
</tbody>
</table>

n.d. = not determined
Ch.6 = results presented in Chapter 6

### The GLUT4 cycle

In *Rationale & hypothesis* the purpose of this work was stated as: to use biochemical and cell biological techniques to better define the proteins and organelles involved in GLUT4 traffic and their possible regulation by insulin. To this end the following major novel findings have been presented in *Chapters 4 through 7*: 1) GLUT4 travels through the early and recycling...
endosomes after internalization and movement into and out of the latter compartment is accelerated by insulin, 2) SNAP-23, VAMP-2 and syntaxin 4 are capable of binding one another, 3) syntaxin 4 is a substrate for various protein kinases \textit{in vitro}, 4) PKA phosphorylation of syntaxin 4 diminishes that SNARE's ability to bind SNAP-23, 5) SNAP-23 specifically associates with a fraction enriched in actin and 6) SNAP-23, VAP-33 and pantophysin function in insulin-stimulated GLUT4 traffic. These findings have led us to propose two models to describe the inter-compartmental traffic of GLUT4 (Figure D-1) and the fusion of GLUT4 vesicles with the plasma membrane (Figure D-2).

As presented in figure D-1 GLUT4 internalizes via a clathrin and dynamin-dependent mechanism at the plasma membrane and arrives at the early endosome. From here it moves into the recycling endosome, a process that is accelerated by insulin and that may involve Rab4. Once in the recycling endosome GLUT4 resides there until recruited to the plasma membrane by insulin. VAP-33 may be involved at this stage, in packaging the vesicles destined for the plasma membrane. The route from the recycling endosome to the plasma membrane remains ill defined as there is insufficient evidence in this study and in the literature to make an unequivocal statement about the existence of a stable GLUT4 storage compartment distinct from the recycling endosome. Insulin's acceleration of movement into and out of the recycling endosome is mediated by PI3K and PKB. The actin cytoskeleton helps to organize events on the right side of the model in figure D-1. An intact actin cytoskeleton is required for movement of PI3K to its sites of action in the exocytic (post-recycling endosome) arc of the GLUT4 cycle and may act as a map to bring vesicles containing GLUT4 areas of the plasma membrane where the two bilayers are able to fuse. A more detailed picture of how GLUT4 vesicles may dock and fuse with the plasma membrane is presented in figure D-2.
In our model of GLUT4 vesicle fusion (Figure D-2) the actin cytoskeleton brings the GLUT4 vesicles into close proximity with the fusion machinery needed to facilitate fusion of those vesicles with the plasma membrane through its linkages to GLUT4 (171) and SNAP-23. In addition to rearranging actin filaments to allow this targeting, insulin also causes the negative regulators munc18c and synip to dissociate from syntaxin 4. In an apparent paradox some reports suggest that munc18c may also be a positive regulator of fusion (119, 140, 345). Pantophysin binds to VAMP-2 and is present on GLUT4 vesicles but its precise role is not known. Once the negative regulators have been removed from syntaxin 4 (and from the other SNAREs?), the v-SNAREs and t-SNAREs form a trans complex, docking the incoming vesicle. Fusion then proceeds, likely in an unregulated manner, resulting in the incorporation of GLUT4 molecules into the plasma membrane.
Figure D-1: The GLUT4 cycle

Once internalized via a clathrin-dependent mechanism GLUT4 vesicles fuse with the early endosome (EE) and, after budding out of the EE, they move on to the recycling endosome (RE). By signalling through PI3K and PKB insulin can accelerate this latter step, perhaps via the action of Rab4. Insulin can also enhance exit of GLUT4 out of the RE, possibly with the help of VAP-33 in the packaging of these exocytic GLUT4 vesicles (EGV). The EGVs, which may or may not be stable, long-lived storage entities, move to the plasma membrane where they can dock and fuse (see Figure D-2). Insulin-dependent actin filament rearrangement (gray mesh) provides underlying organization to the signalling and EGV targeting.
Figure D-2: GLUT4 vesicle fusion

In the basal state both munc18c and synip are bound to syntaxin 4 while SNAP-23 and GLUT4 are associated with the actin cytoskeleton. Pantophysin and VAMP-2 are located on GLUT4 vesicles. Insulin causes the formation of actin ruffling and the dissociation of munc18c and synip from syntaxin 4. Once brought into proximity of the fusion machinery at the plasma membrane by the cytoskeleton, the GLUT4 vesicle can dock and subsequently fuse through the action of syntaxin 4, SNAP-23 and VAMP-2 resulting in the exposure of GLUT4 to the extracellular milieu.
References


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Appendix A – Other work

During the course of this thesis work I was involved in numerous other projects that are not described here because they remained inconclusive. Some of these projects include:

1. Examination of SNAP-23 in polymorphonuclear leukocytes (PMN). In collaboration with many other members of our laboratory we examined the distribution of SNAP-23 in PMN in relation to syntaxins 2, 3 and 4.

2. Binding characteristics of SNAP-23B. One group who cloned SNAP-23 also found a splice variant of this protein lacking the polycysteine palmitoylation motif. This splice variant was termed SNAP-23B (229). In collaboration with Dr. Zhi Liu, a molecular biology technician, and Brian Yeung, a summer student under my supervision, we constructed this variant using polymerase chain reaction. We looked for the existence of this protein in 3T3-L1 adipocytes before continuing with further experiments but we could find no evidence that SNAP-23B was expressed in this cell line. No other investigators have found SNAP-23B protein expression (127).

3. Dynamin phosphorylation. In parallel with the in vivo SNARE phosphorylation experiments discussed in Chapter 5 we searched for increases in dynamin II phosphorylation in response to insulin. We found that insulin could enhance dynamin phosphorylation in a wortmannin-independent manner. Dynamin phosphorylation could be pursued through the use of mass spectrometry to identify the site(s) of phosphorylation and quantify the extend of phosphorylation in response to insulin.
4. Function of endophilin and synaptojanin in GLUT4 endocytosis. Using reagents obtained from Dr. Pietro De Camilli at Yale University, we injected fragments of endophilin 2 and synaptojanin 1 into 3T3-L1 adipocytes and measured their effect on GLUT4 internalization. Peptides from both proteins inhibited GLUT4 internalization. This work will be pursued further by another student/fellow.