[Frontiers in Bioscience 8, d620-641, May 1, 2003]

SNARES for GLUT4 – MECHANISMS DIRECTING VESICULAR TRAFFICKING OF GLUT4

Julian Grusovin and S. Lance Macaulay

CSIRO Health Science and Nutrition, 343 Royal Parade, Parkville, Australia 3052

TABLE OF CONTENTS

1. ABSTRACT

Fusion of GLUT4 vesicles with the plasma membrane is a key terminal step in insulin-regulated glucose transport. This fusion event is mediated by SNARE proteins, syntaxin 4, SNAP23 and VAMP2, through a process regulated by accessory proteins whose roles are still unclear. Munc18c is a key regulatory protein of syntaxin, and possibly of whole-SNARE complex cycling. Rab GTPases, through interactions with tethering molecules and SNAREs, provide targeting specificity in the GLUT4 secretory pathway. We review here insulin-mediated signaling of glucose transport, and particularly the role played by SNAREs and their accessory proteins.

2. INTRODUCTION

The maintenance of blood glucose at the homeostatic concentration is essential to whole-body well being, and is a major physiological activity of insulin and glucagon. After a meal, and in response to elevated blood sugar levels, pancreatic β- cells secrete insulin that, in a healthy individual, suppresses hepatic glucose output (gluconeogenesis) and stimulates glucose uptake in the insulin-responsive tissues, skeletal muscle and fat. Non-insulin dependent diabetes mellitus (NIDDM) develops from insensitivity of these tissues to insulin, and resultant chronic and abnormally high levels of blood glucose. This impairment contributes significantly to the complications of the disease that include renal failure, retinopathy, and nerve and microvascular tissue damage. The partitioning of glucose out of blood and into cells is therefore critical, and is the job of a family of facilitative glucose transporters (GLUTs); integral membrane proteins with predicted twelve membrane-spanning helices. Of particular importance to insulin action is GLUT4, a transporter largely restricted to the insulin-responsive tissues, fat, and skeletal and cardiac muscle (1, 2). Under basal conditions, GLUT4 is localized mainly to intracellular membrane compartments. After a meal, and in response to cellular insulin stimulation, GLUT4 is rapidly redistributed from intracellular compartments to the plasma membrane (2,3,4) where it facilitates uptake of glucose into the cell.

The correct delivery of GLUT4-containing vesicles to the plasma membrane and the recycling of GLUT4 through intracellular membrane compartments relies, in part, on the recognition of target membranes by the vesicle and correct couplings of proteins that direct the fusogenic process. These proteins, called SNAREs (Soluble NSF attachment protein receptors), form a class of coiled-coil proteins associated with the fusing membranes and, in the case of GLUT4 exocytosis, are exemplified by VAMP2 (vesicle-associated membrane protein 2) on GLUT4 vesicles, and by syntaxin 4 and SNAP23 on the plasma membrane (Table 1). Animal models such as the Zucker fatty rat that expresses abnormal SNARE levels (5), and syntaxin 4 heterozygous mice that develop muscle insulin

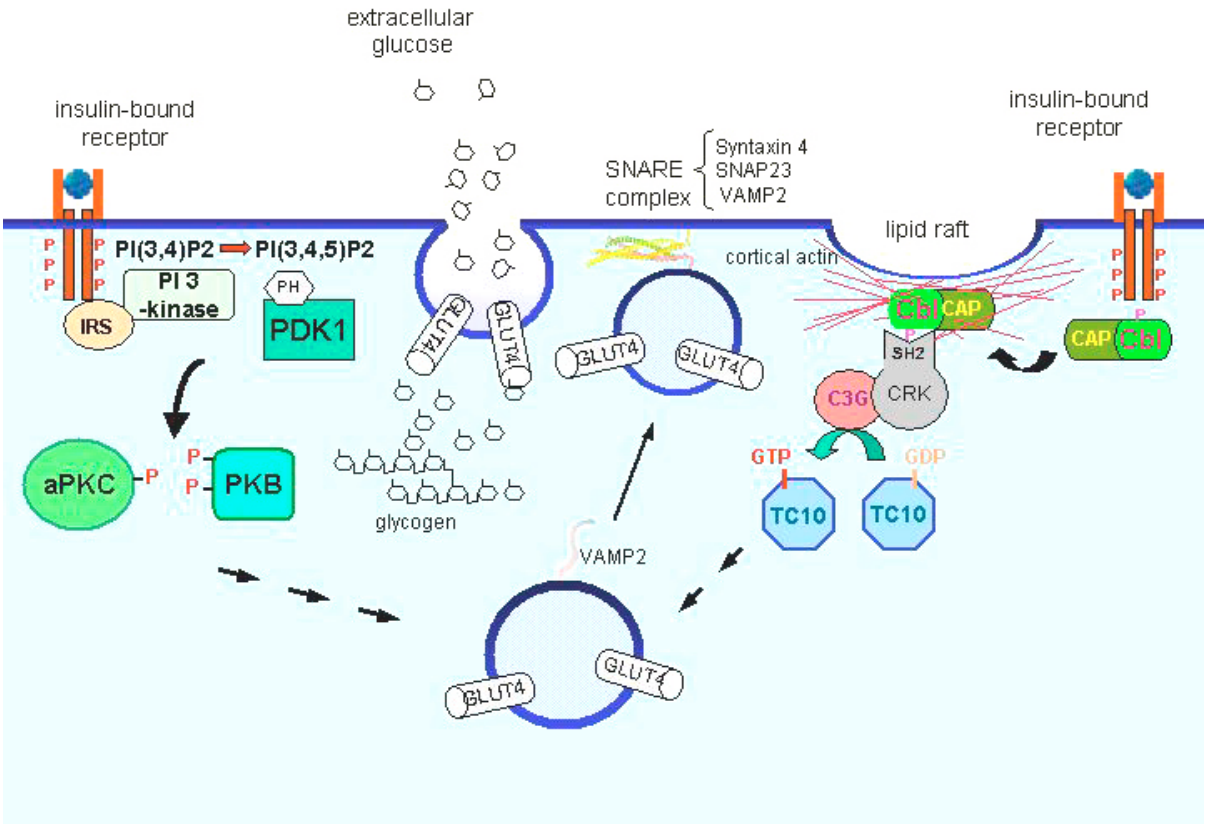

Figure 1. Insulin signaling pathways mediating glucose transport. Two discrete pathways have been implicated in insulin-stimulated glucose transport. One is mediated via IRS and PI 3-kinase activation (left side), and the other by c-Cbl/CAP (right side). Both pathways are needed to stimulate GLUT4 translocation in adipocytes. Insulin binding to its receptor causes a conformational change in the receptor leading to activation and autophosphorylation of its cytosolic tyrosine kinase. Tyrosine 960 acts as a docking site for IRS which is phosphorylated by the receptor and binds a number of substrates including PI 3-kinase. PI 3-kinase phosphorylates inositol phospholipids in the 3' position enabling recruitment, phosphorylation and activation of PDK1 and its associated substrates PKB and atypical PKC's zeta and lambda (aPKC) via association of its PH domain. Downstream targets of these kinases resulting in GLUT4 translocation are unclear. On the right hand side, insulin-stimulated phosphorylation of CAP/Cbl triggers its association with lipid rafts via flotillin, where phosphorylated Cbl recruits the adaptor protein CrkII and C3G. C3G is a guanine nucleotide exchange factor for the GTPase, TC10. The terminal signaling steps of this GTPase activity mediating GLUT4 translocation are not yet known.

resistance (6) underscore the importance of SNAREs to glucose homeostasis.

In this review we briefly recapitulate the itinerary of insulin signaling events that lead to GLUT4 vesicle mobilization and the compartmentalization of GLUT4 itself. Particular attention is then paid to the development of the SNARE hypothesis, to the major players relating to the final stages of GLUT4 vesicle exocytosis and to molecules that may regulate this event.

3. INSULIN RECEPTOR SIGNALING TO GLUT4

Insulin exerts its action on a wide variety of responsive tissues both as a growth factor and as a metabolic regulator, affecting carbohydrate, lipid and protein metabolism (7). The cellular response to insulin is mediated by its cell-surface insulin receptor (IR), a disulphide-linked, homodimeric glycoprotein consisting of two extracellular alpha-subunits, and two membrane-spanning beta-subunits that possess intracellular tyrosine kinase activity (Figure 1) (8, 9,10). Insulin binding to the alpha-subunit activates the tyrosine kinase domain by inducing autophosphorylation, in *trans*, of Tyr1162, Tyr1163 and Tyr1158 in the juxtaposed activation loop (11), as well as other tyrosines. Some of these, phosphotyrosine960 in particular, serve as recognition signals for binding of a range of adaptor proteins, including the insulin receptor substrate family (IRS1-4), Cbl, Gab1 and Shc. When these proteins are phosphorylated by the insulin receptor kinase, they act as docking sites for downstream effectors that contain SH2 or PTB binding sites, such as phosphatidylinositol 3-kinase (PI 3-kinase) and Grb2/SOS.

Table 1. SNAREs and accessory proteins

Protein	Description	Example yeast homologues	Key Reference
NSF	N-ethylmaleimide-sensitive factor, soluble ATPase involved in fusion	Sec18p	104,124
SNAP	Soluble NSF attachment protein, binds NSF, three isoforms	Sec17p	104,124
t-SNARE	Target membrane soluble NSF attachment protein receptor		
v-SNARE	Vesicle membrane soluble NSF attachment protein receptor		
syntaxin	t-SNARE enriched on target membranes, many isoforms. Syntaxin 4 is GLUT4 plasma membrane t-SNARE also involved in other fusion events	Sso1p, Sso2p, Sed5p, Tlg2p	6,111, 181
SNAP23	t-SNARE enriched on target membranes, t-SNARE for GLUT4 and other fusion events, three isoforms,	Sec9p	111,181
VAMP	v-SNARE, vesicle associated membrane protein, many isoforms. VAMP2 is insulin-responsive GLUT4 vesicle SNARE also involved in other fusion events	Sec22p, Snc1p, Bet1p	111,181
Munc18	Syntaxin binding protein implicated in fusion, three isoforms. Munc18c binds syntaxin 4, involved in GLUT4 trafficking and other fusion events.	Sec1p, Vps45p, Sly1p, Slp1	187
Rabs	Low molecular weight GTPases involved in vesicle traffic and possibly tethering, many isoforms	Sec4p, Ypt1	210
Synip	Syntaxin 4 interacting protein		169
Pantophysin	Synaptophysin homologue, binds VAMPs		159
VAP-33	VAMP-associated protein		157

Recruitment of PI 3-kinase by IRS leads to its activation (12) and facilitates the phosphorylation of phosphatidylinositol(4,5)bisphosphate ($PI(4,5)P_2$) to $PI(3,4,5)P_3$. Reagents that block this step, such as the fungal metabolite wortmannin (13) or LY294002, also block insulin-stimulated GLUT4 translocation (14) and glucose transport in adipocytes (15) and muscle (16, 17, 18). The effect of wortmannin on insulin-stimulated cells can be overcome by the addition of membrane-permeant esters of $PI(3,4,5)P_3$, suggesting that the activity of PI 3-kinase is essential to the metabolic function of insulin (19).

Attractive candidates for mediating the effects of $PI(3,4,5)P_3$ in insulin-responsive cells are members of the AGC superfamily of serine kinases: phosphoinositide dependent protein kinase-1 (PDK1) and its substrates, protein kinase B (PKB, also known as Akt), and the atypical protein kinase Cs (aPKC), zeta and lambda (20, 21, 22,23)(Figure 1).

PDK1 is activated upon binding of its PH domain to $PI(3,4,5)P_3$ (20). This kinase, in turn, phosphorylates and activates PKB/Akt isoforms as well as aPKCs. Recent data suggests that PKB is localized to the plasma membrane for activation by its association with PDK1 (24), although both PKB and aPKCs have pleckstrin homology (PH) domains that may also participate in directing their membrane association. PKB exists in three isoforms that appear to be differentially expressed in different tissues. Both 3T3L1 and rat adipocytes preferentially express PKBbeta (25, 26), whereas PKBalpha is more highly expressed in rat hepatocytes (27). Insulin-stimulated translocation of PKBbeta to the plasma membrane has been demonstrated in several studies (28, 26). Two further studies have reported the association of PKBbeta with GLUT4 vesicles (29, 30), suggesting that PKBbeta can link insulin signaling with glucose transport. Consistent with this suggestion, it has been demonstrated that targeting kinase-inactive PKB to GLUT4 vesicles markedly inhibits GLUT4 translocation (31), and overexpression of constitutively active PKB or PDK1 stimulates GLUT4 translocation (32, 33), whereas microinjection of cells with antibodies directed against PKB, or with a substrate peptide, inhibits insulin-stimulated GLUT4 translocation (26). Finally, PKBbeta-knockout mice, but not those ablated in PKBalpha, display insulin resistance and defects in insulin action in liver and muscle (34,35).

In addition to activating PKB, PDK1 also phosphorylates the activation loop of the atypical PKCs zeta and lambda (23), proteins implicated by several studies as effectors of insulin-stimulated GLUT4 translocation. Overexpression of aPKCs in human or rat adipocytes (33,36), in rat skeletal muscle (37) or in 3T3-L1 adipocytes (38) stimulates plasma membrane association of GLUT4 and glucose uptake. Impaired activity of aPKC is also associated with insulin resistance and reduced glucose uptake in skeletal muscle of obese patients (39) as well as impaired GLUT4 translocation in fat-fed rats (40). A clear understanding of the relative roles of PKB and PKC in insulin-stimulated glucose transport is yet to emerge, however a recent study has provided some evidence that PKB and aPKCs are able to form signaling complexes (41).

While these $PI(3,4,5)P_3$ -mediated kinase activities are clearly necessary for the metabolic effects of insulin, several lines of evidence suggest that they alone are not sufficient to elicit the full metabolic response. For instance, the very same esters of $PI(3,4,5)P_3$ that can substitute for PI 3-kinase activity cannot, in the absence of insulin stimulation, induce glucose uptake (19). Similarly, neither PI 3-kinase activation by PDGF (42) nor overexpression of constitutively active PI 3-kinase results in glucose uptake to levels achieved by insulin stimulation (43), pointing to additional pathways regulating insulin-stimulated glucose uptake.

One potential pathway involves the small GTPase, TC10 (Figure 1). In adipocytes, activated insulin receptor directly phosphorylates Cbl complexed with CAP (the Cbl-associated adaptor protein) and the adaptor protein APS (44), whereupon the complex is recruited by flotillin and relocates to caveolin-enriched lipid rafts (45). Activated Cbl then recruits the CrkII-C3G complex to lipid rafts, where the nucleotide exchange factor C3G specifically activates the small GTP-binding protein TC10 (46). This pathway is independent of the PI 3-kinase pathway, but insulin-stimulated glucose transport can be arrested by expression of CAP N-terminal domain (45) or of the dominant-negative T31N mutant of this GTPase (46, 47), indicating the potential importance of this pathway in insulin-stimulated glucose transport in adipocytes. Interestingly, studies in our laboratory have not been able to demonstrate this pathway in skeletal muscle (Wadley, unpublished data). TC10 may exert its activity through the cytoskeleton, since the inhibitory TC10/T31N mutant that inhibits insulin stimulated GLUT4 translocation and glucose transport also inhibited insulin-induced cortical localization of N-WASP and F-actin formation (48). Furthermore, expression of a constitutively active mutant, TC10/Q75L, which causes perinuclear actin polymerization, also disrupted cortical actin polymerization and GLUT4 trafficking, similar to the TC10/T31N mutant (49). Consistent with these data, treatment of cells with the actin stabilizer, jasplakinolide, resulted in a dose-dependant inhibition of insulin-stimulated GLUT4 translocation, suggesting that actin remodeling is necessary for GLUT4 translocation (50). The TC10-interacting protein CIP4/2 (51) has been reported to interact with WASP and to mediate its interaction with microtubules in COS cells (52). However, the interaction between CIP4/2 and WASP could not be detected in adipocytes (51). A novel caveolin-associated actin structure “cav-actin” has been observed in adipocytes and proposed to play a role in the TC10 pathway (53). However, these novel structures have also been interpreted as large invaginations or “caves” containing all elements of the plasma membrane, including caveolae, clathrin-coated pits and an associated cortical actin network (54). Notably, GLUT4 is recruited to these structures in response to the insulin stimulus.

In addition to the PI 3-kinase and TC10 pathways that mediate GLUT4 translocation, several studies suggest that GLUT4 requires additional activation, further to its translocation to the plasma membrane, to make it fully competent to transport glucose into the cell. This activation may involve p38 MAP kinase, as an inhibitor of p38 MAPK inhibits glucose transport into adipocyte and muscle cells without affecting GLUT4 translocation (55). Furthermore, a differential dose dependency of the effect of wortmannin on insulin-stimulated glucose transport and GLUT4 translocation has been detected in 3T3-L1 adipocytes and L6 myotubes (56, 57). Translocation of GLUT4 is inhibited at concentrations of wortmannin that also inhibit PKB and aPKC, whereas glucose transport, but not GLUT4 translocation, is inhibited at much lower concentrations (57). The biochemical mechanism underlying this aspect of GLUT4 activation is unclear.

How these various PI 3-kinase-dependent and -independent signaling pathways converge on GLUT4 translocation and glucose transport remains an open question. Notwithstanding, this bifurcation may impose a higher level of specificity and acuity of response than any single pathway; for example, the PI 3-kinase pathway, which can also be activated by receptors other than the insulin receptor.

4. GLUT4 TRAFFICKING COMPARTMENTS

The biochemical basis of GLUT4 translocation is as much a function of cellular localization as it is of the signaling cascades discussed above. In this context it is important to consider the nature of the cellular compartments to which GLUT4 is localized, and the proteins that characterize them.

Immunocytochemical analysis suggests that in adipocytes and myocytes under basal conditions, most of the GLUT4 pool is localized to small vesicles, to tubulo-vesicular structures associated with the *trans*-Golgi network (TGN), near the plasma membrane, and scattered, or in clusters, throughout the cytoplasm (58,59,60), as well as to vacuoles (61). Insulin stimulation leads to a reduction of GLUT4 from the small vesicle population, with a ~10-40-fold increase in the level found in plasma membrane, a small increase within recycling structures such as early endosomes and coated vesicles in adipocytes (59, 62, 61), and a translocation to the T-tubule system and the sarcolemma in muscle (59, 63, 60).

Considerable evidence suggests the existence of a specialized insulin-sensitive compartment - the GLUT4 storage vesicle (GSV) - which is morphologically separate from the general endosomal system, and is characterized by the vesicular colocalisation of the cargo proteins GLUT4 and IRAP (insulin-responsive aminopeptidase) as well as the v-SNARE, VAMP2 (Figure 2). The GSV forms a subset of the low-density microsomal (LDM) fraction obtained from differential centrifugation of adipocyte homogenates (64). Immunocytochemical analysis (61), and chemical ablation of the LDM (65, 66) demonstrate the existence of a population of vesicles that comprise about 60% of the GLUT4 pool, and which also contain VAMP2. This GSV pool is largely devoid of markers of early and late endosomes as well as of lysosomal markers, and is readily mobilized to the plasma membrane in response to insulin (67). Conversely, a significant proportion of GLUT4 is colocalized with cellugyrin (68) and with the mannose 6-phospate receptor (MPR), a marker of TGN/endosomal cycling. This pool largely excludes VAMP2 (61). Surface-labeling experiments suggest that the GLUT4/MPR pool does not exocytose extensively under basal conditions (69), while cellugyrin maintains intracellular localization both in the presence and absence of insulin (68). It thus appears that cellugyrin is excluded from the insulin-responsive GLUT4 pool but is represented on vesicles trafficking between early endosomes and recycling endosomes, while MPR may penetrate the GLUT4 compartment non-specifically (68).

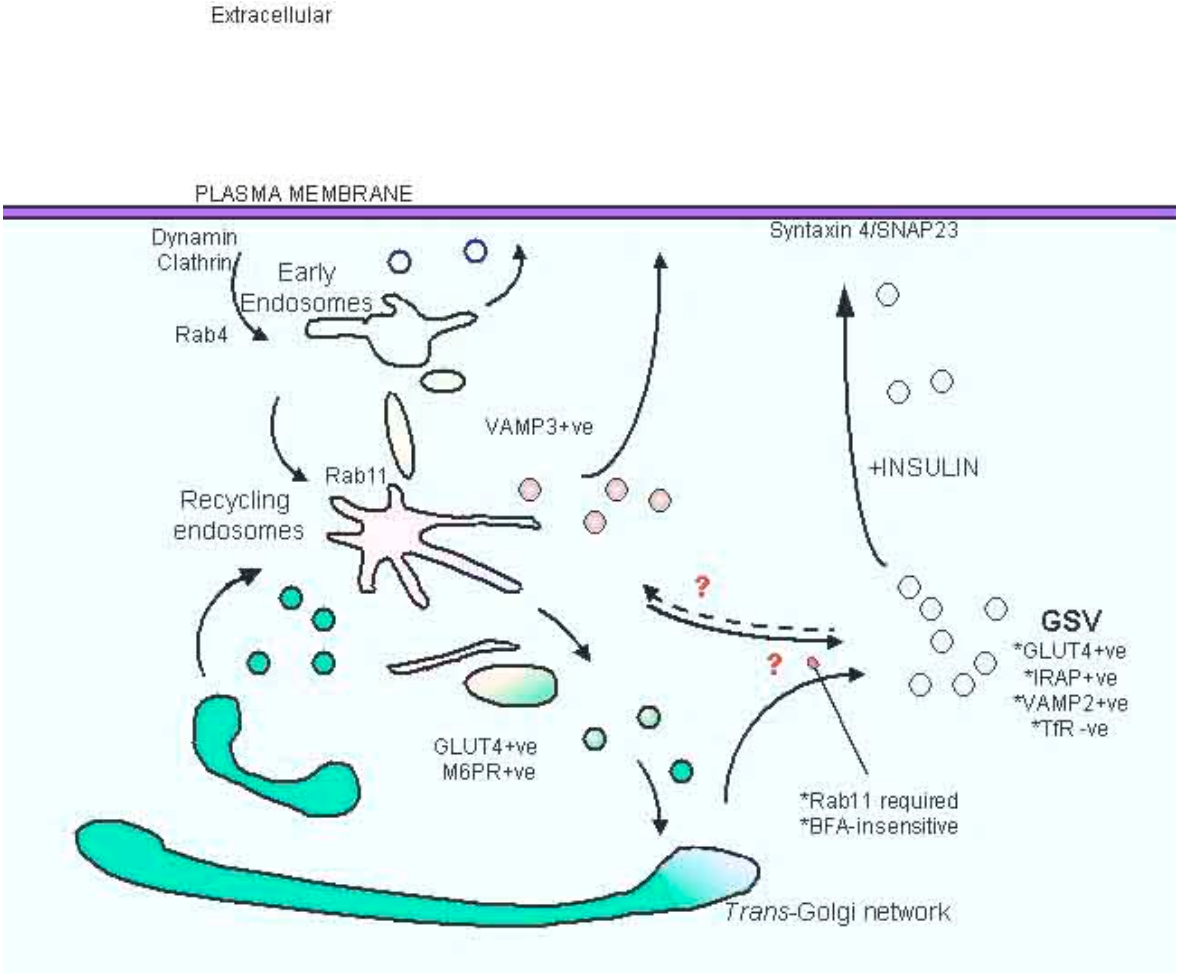

Figure 2. A possible trafficking itinerary for GLUT4. GLUT4 occurs in an insulin-responsive pool, and also as a constituent of M6PR- and TfR-containing vesicles. Under basal conditions, the GSV appears to be relatively static and excludes markers of constitutive recycling. The non-GSV GLUT4 pool may cycle between the TGN and endosomal systems, and is exposed to recycling markers. Insulin stimulates trafficking from all GLUT4 compartments including both the VAMP2- and VAMP3-positive pools. GLUT4 recycles in a clathrin-dependent fashion from the plasma membrane to the early endosomes. It is uncertain whether the GSV arises from the TGN or from the recycling endosomes, though its formation is subject to a Rab11-dependent step. It is also unclear if the GSV compartment represents a population of tethered vesicles or a compartment in dynamic communication with the endosomal or TGN system.

The genesis of the GSV remains unresolved. It has been proposed that this specialized compartment arises from the TGN, cycling to the recycling endosome compartment under basal conditions, but to the cell surface upon insulin stimulation (Figure 2) (70). The presence of the MPR marker on GLUT4 vesicles supports this model, however only a small proportion of this pool contains VAMP2 (61). Moreover, treatment of cells with brefeldin A, which destroys Golgi compartments, does not prevent the genesis of the GSV compartment (71). An alternative model has been developed that is consistent with the observation that GSVs are a post-endosomal compartment (72). This model suggests that GSVs derive from recycling endosomal compartments (73), and is supported by a study showing that a dominant-negative Rab11 inhibitor blocks formation of the GSV, and withdraws GLUT4 into a concentrated perinuclear endosomal compartment which also contains the transferrin receptor (73).

Interestingly, GLUT4 itself may be a determinant of the formation of the GSV. One study has shown that homozygous GLUT4-knockout mice fail to sequester and traffic IRAP in the same way as in wild-type mice. Rather, IRAP is trafficked constitutively and in an insulin-independent fashion through the endosomal system (74). This is noteworthy because IRAP and GLUT4 normally co-habit the same vesicle and traffic together in an indistinguishable manner (75, 76). The failure of GLUT4-knockout mice to develop an insulin-regulated IRAP compartment suggests the possible existence of a molecular signature on the GLUT4 protein that is essential for the formation of the GSV/IRAP compartment.

Conversely, IRAP knockout mice do not exhibit an altered sub-cellular localization of GLUT4, but the levels of expressed GLUT4 are diminished (77). However, while GLUT4 may be necessary for GSV formation, it is not by itself sufficient, since C2C12 cells, which express both GLUT4 and IRAP, lack this compartment (78).

On exposure to insulin, GLUT4 in adipocytes and muscle is translocated to the plasma membrane. GLUT4 on the plasma membrane is not distributed in a random fashion (58), and the nature of this uneven disposition, as well as the mechanism of GLUT4 internalization on removal of the insulin stimulus have been the subject of considerable investigation. Several functional studies suggest that GLUT4 endocytosis from the plasma membrane is mediated by a dynamin- and clathrin-dependent process (79, 59,80, 81), but these studies appear to be at odds with the proposed association of GLUT4 with caveolae and a suggested caveolae-mediated internalization mechanism (82, 83). However, recent microscopical studies dispute the morphological basis for a specific GLUT4-caveolae association (58, 54). Internalized GLUT4 traffics rapidly to the early endosome (84) before progressing to the perinuclear recycling endosome. Interestingly, insulin treatment hastens the passage of GLUT4 through the recycling system, without affecting the rate of transferrin receptor endosomal cycling (84). Aside from the involvement of Rab GTPases and molecular targeting motifs on GLUT4 itself (85), studies of the molecular basis of the endomembrane traffic of GLUT4 remain poorly developed.

A number of studies have examined the involvement of the microtubule network in directing trafficking of GLUT4. Studies with the microtubule depolymerizing drug, nocodazole, show that treatment of cells with concentrations which completely depolymerize microtubules and which disperse the Golgi apparatus and perinuclear localized GLUT4, has no effect on insulin-stimulated glucose uptake or GLUT4 translocation (86,87). In contrast, and at elevated concentrations of nocodazole, glucose uptake but not GLUT4 translocation is inhibited (86,87). Microtubule depolymerization also restricts newly endocytosed GLUT4 to a domain just below the plasma membrane and from which it is unable to undergo further sorting (86). These data are consistent with a role for dynein and the microtubular network in the internalization of GLUT4 to the perinuclear compartment in a Rab5- and PI 3-kinase-dependent manner (88). Thus, while internalization and sorting of GLUT4 through to the perinuclear region of the cell may involve the microtubular network, it does not appear necessary for the rate-limiting step in exocytosis of GLUT4 or the early stages of endocytosis. Real-time confocal studies have shown insulin to stimulate long-range movement of GLUT4 vesicles along linear tracks in adipocytes (89). It would be of interest to repeat these studies in the presence of nocodazole as this may shed light on the rate-limiting exocytic localization of GLUT4.

As has been discussed, the cortical actin cytoskeleton appears to be a target of signaling by the TC10 pathway. However, it is also clear that GLUT4 itself colocalizes with regions of cortical actin assembly. Consistent with a role for the actin cytoskeleton in Glut4 trafficking, actin depolymerizing agents inhibit GLUT4 exocytosis, but interestingly, not endocytosis (90, 91).

5. VESICLE TARGETTING AND FUSION

5.1. Development of the SNARE Hypothesis

While the details of the regulatory mechanisms involved in translocating GLUT4 to the plasma membrane remain unclear, much has been learned about the fusogenic process, and this forms the major focus of the remainder of this review. Thus far, much of our current understanding of the process, and the machinery involved, has come from the delineation of membrane trafficking processes in other cells types, particularly of neurotransmitter release from neuronal cells and secretion in yeast. Indeed, the seminal work of Rothman, Scheller, Schekman and co-workers has provided a basis for understanding regulated trafficking of transport vesicles through the compositionally distinct membrane compartments of the cell. This regulation enables the correct sorting of cargo destined for traffic, the targeting of nascent vesicles carrying this cargo, and recognition and fusion of these vesicles at their correct destinations. It is therefore of value to consider first the development of our current understanding of the process of regulated vesicle fusion and protein trafficking, and then to consider the proteins that are involved in GLUT4 trafficking.

The first major inroad in elucidating the molecular machinery responsible for membrane fusion came with the development of *in vitro* assays that sought to reconstitute traffic between two populations of discreet Golgi cisternae deficient in characteristic glycosyltransferases. By following the pattern of glycosylation of a secreted viral protein, it was demonstrated that correct oligosaccharide processing could only occur by enzymatic complementation between the two sets of Golgi.

This series of studies provided compelling evidence that trafficking between cisternae was a process involving a diffusible transport vesicle, allowed the resolution of sequential intermediate steps in the process, and shed light on the requirements for cytosolic factors (92, 93, 94, 95).

The inhibition of fusion by N-ethyl maleimide (NEM), and the ability to reconstitute it by the addition of cytosolic fractions, allowed the catalytic NEM-sensitive fusion protein (NSF) to be isolated and characterized (96, 97). Remarkably, NSF was shown to be a homologue of yeast Sec18p (98), a protein known to be essential in ER-to-Golgi traffic and identified from a screen of yeast secretory mutants. However, NSF alone was insufficient to reconstitute membrane fusion, but extending this reconstitution approach led to the isolation of the family of Soluble NSF Attachment Proteins (alpha-, beta- and gamma-SNAPs) (99), functional homologues of the yeast secretory protein Sec17p (100). In yeast strains in which

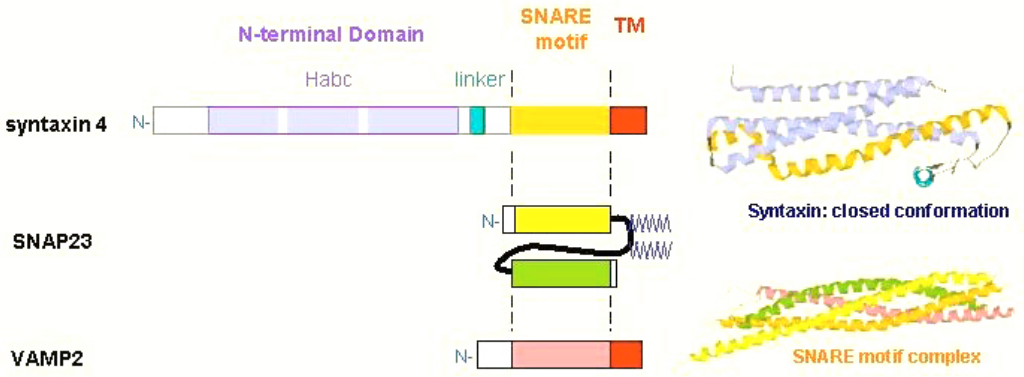

Figure3. Schematic representation of SNAREs. The SNARE motifs for syntaxin 4 are shown in orange, for SNAP23 in yellow and green, and for VAMP2 in salmon. Transmembrane (TM) regions for each SNARE protein are shown in red. The syntaxin N-terminal regulatory domain is structured at all times. Formed SNARE complexes are well structured and extraordinarily stable, forming helical bundles that are resistant to disassembly by heat or detergent. Individual motifs are color-coded throughout. The SNAP23 geranylgeranyl groups are represented by the blue "springs" Molscript graphic schematic of SNAREs modified from (113, 166).

either of these genes was present as a temperature-sensitive mutant, culturing at the non-permissive temperature blocked trafficking to the Golgi and led to the accumulation of vesicles (101). These two molecules thus appear to be functionally and structurally conserved in eukaryotes, suggesting a common apparatus for membrane trafficking.

Correct fusion of a vesicle to its destination membrane is implicit in all membrane trafficking events. The notion that specificity is intrinsic to the fusing membranes themselves is supported by studies in both mammals (102) and yeast (100), which established that the association of NSF/sec18 and SNAP/sec17 with membranes required an integral membrane SNAP receptor. A large, detergent-stable 20S complex which dissociates in an ATP-dependent manner and which consists of NSF, SNAP and putative membrane receptors was subsequently identified (103). This complex was purified from the gray matter of bovine brain using tagged NSF and recombinant alpha- and gamma-SNAP (104), and four SNAP receptors (SNAREs) were characterized (104). These SNAREs had previously been identified as synaptosomal proteins: syntaxins A and B (105) as plasma membrane-anchored docking proteins for synaptic vesicles, VAMP/synaptobrevin (106, 107) as an integral membrane protein of small synaptic vesicles, and SNAP25 - the 25kD synaptosomal-associated protein (108). Thus, the 20S particle provided a nexus between these three classes of synaptosomal proteins as well as with the catalytic activity of NSF/SNAPs. These receptors were classified on the basis of their membrane of origin (vesicle) v-SNARE or (target membrane) t-SNARE.

Underpinning the idea that SNAREs may lie at the heart of most membrane trafficking events, including that of GLUT4, was the discovery of a large family of related SNARE molecules, including SNAP23 (109) and syntaxins 2-5 (110, 111), as well as the earlier identification of VAMP as a constituent of GLUT4 vesicles isolated from 3T3-L1 adipocytes (112).

5.2. The Structure And Function of SNAREs

SNAREs are a structurally variable class of proteins ranging in size from ~17 kDa for VAMPs to ~40 kDa for syntaxins (Figure 3). A unifying and evolutionary conserved feature of SNAREs, which is central to their function, is the possession of a ~60 residue SNARE motif capable of forming quaternary coiled-coil complexes with other SNARE motifs (113, 114, 115). In most cases of vesicle fusion at endomembranes, the four SNARE motifs are drawn from four individual SNARE molecules to form the quaternary complex. However, for exocytic SNARE complexes formed at the plasma membrane, two of the four motifs are contributed by SNAP23 or equivalent isoforms (111). An interesting exception is homotypic vacuole fusion in yeast, which appears to require five SNAREs to form a pentameric complex (116). In all instances thus far examined, at least two SNARE molecules, one contributed by each fusing membrane, reside as integral membrane proteins with the SNARE motif proximal to the lipid bilayer and the residual N-terminal domain extending further into the cytoplasm.

With the exception of members of the SNAP23/25 family, a common fold for the N-terminal domain of t-SNARES appears to be a three-helix bundle, the Habc domain, (117,118) (Figure 3). The Habc domain of exocytic syntaxins 1 and Sso1p can fold back on the SNARE motif and form a "closed" conformation, whilst endomembrane t-SNAREs syntaxin 8, vti1b and VAM3p

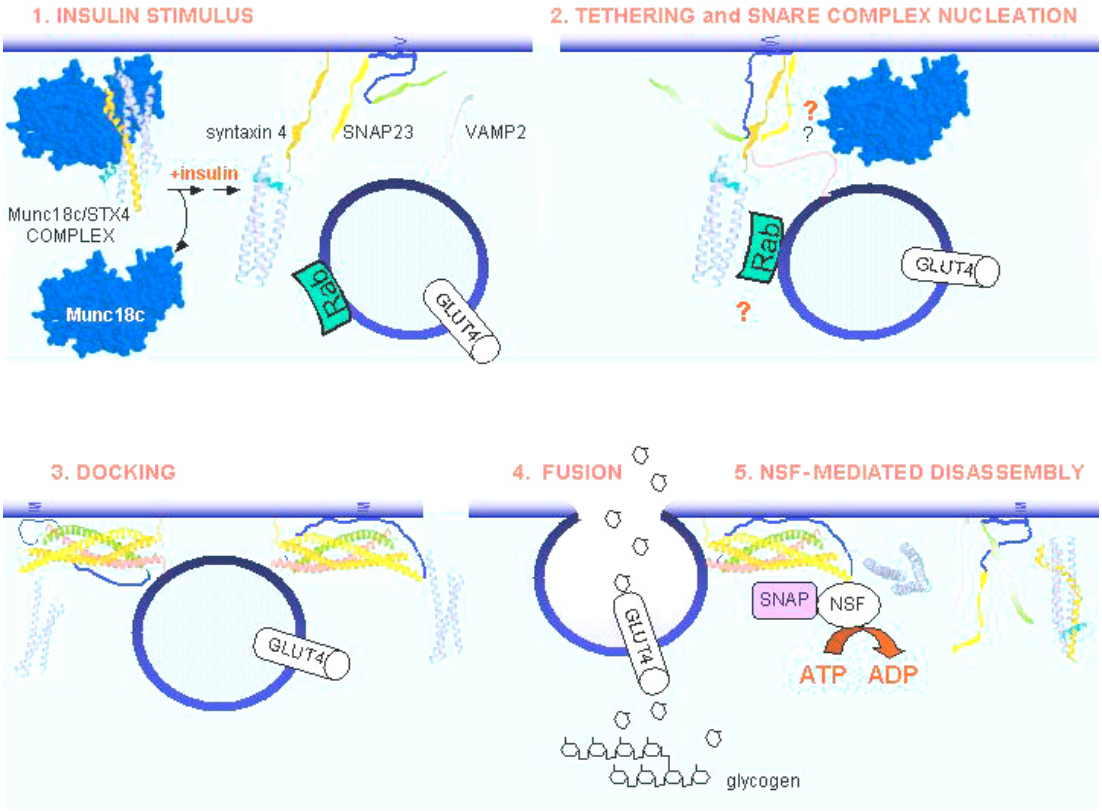

Figure 4. Hypothetical steps mediating GLUT4 vesicle fusion. 1. Insulin stimulates the recruitment of GLUT4 vesicles to the plasma membrane, as well as the dissociation of Munc18c from syntaxin 4. 2. Rab proteins may tether the vesicle to the plasma membrane, possibly via syntaxin 4. 3. SNARE motifs on opposing membranes nucleate to form a *trans*-SNARE complex, and "zippering-up" is favoured thermodynamically. SM proteins may possibly play a "proof-reading" or other facilitatory role at this late stage. 4. Sufficient free-energy may be released during SNARE complex formation to drive membrane fusion. 5. Dissociation of the resultant *cis*-SNARE complex is energy-dependent and carried out by NSF.

have been observed only in the open conformation (119, 120, 115). The closed conformation of syntaxin may be a regulatory mechanism to limit the availability of the SNARE motif for *trans* complex formation with the v-SNARE, but the mechanism by which syntaxin opens to expose the SNARE motif is not understood. It is thought that the formation of the quaternary SNARE complex, in *trans*, between SNARE motifs bridges the opposing membranes, and the free energy released as they "zip up" to form a parallel bundle (113, 114) may be sufficient to force fusion by driving the membranes together (Figure 4). However, some reports suggest that SNARE complex formation does not lead directly to membrane fusion. For instance, yeast Sec1p (the Munc18 homologue) is reported to associate with pre-formed SNARE complexes prior to fusion (121) and homotypic fusion of the yeast vacuole is insensitive to *trans*-SNARE complex disassembly (122), suggesting that other factors may operate downstream of SNARE complex formation.

The SNARE complex, as isolated by Sollner and colleagues (104), consists of syntaxin 1, VAMP and SNAP25 and appears to represent the low-energy form of the SNARE complex. In this form, SNAREs are extraordinarily stable, with high melting temperatures and, to varying degrees, are resistant to denaturation by SDS (123). In order for the molecules in the *cis*-SNARE complex to be functionally competent to undergo further rounds of fusion, the individual components must dissociate and be recycled to their correct membrane population. Thus, it is the low-energy SNARE complex that constitutes the physiological target of NSF/SNAP, as indicated by its ATP-dependent disassembly in the presence of this catalytic complex (124).

5.3. Specificity of SNARE Coupling

The specific pairing of SNAREs has been proposed to be essential for the fidelity of membrane recognition and fusion (104). However, an exhaustive *in vitro* analysis of the couplings of isolated mammalian SNARE motifs has shown that these interactions, rather than being selective, are quite promiscuous (123), suggesting that membrane fusion specificity is not encoded by SNARE molecules. Indeed, the t-SNAREs that mediate GLUT4 exocytosis, syntaxin 4 and SNAP23, form more stable and SDS-resistant complexes with VAMP4 and VAMP7 than with the cognate VAMP2 (123).

This apparent lack of specificity may hold advantages for an organism by providing redundancy; for example, in cases where a particular SNARE is no longer functional. In support of this notion, yeast v-SNARE Ykt6p is upregulated in, and can compensate for the loss of Sec22p function in deletion mutants (125); VAMPs in Drosophila are functionally interchangeable (126); and Vti1p in yeast functions in two different pathways (127).

Notwithstanding the potential for a certain degree of redundancy, a series of studies of *in vitro* membrane fusion mediated by specific SNARE pairs have provided evidence that, for some yeast SNAREs at least, and probably in general, specificity may be largely preserved if SNAREs are membrane-bound and in very specific combinations, reflecting the pattern of membrane flow found in the cell (128, 129). Clearly, elements of the above studies are in contradiction and the extent to which *in vitro* assays accurately reflect SNARE pairings *in vivo* is not yet clear.

Vesicles that traffic to sites of secretion appear to be tethered prior to, and independent of, SNARE complex formation. This process may affect both specificity of docking and favor SNARE pairing. Peripheral membrane factors such as the sec6/sec8 complex (130), p115/giantin/GM130 (131, 132) and EEA1 have all been implicated as tethering agents (133). A common feature of many of these tethering systems is their interaction with, and possible regulation by, Rab GTPases and their effectors (134).

5.4 SNAREs Mediating GLUT4 Traffic

5.4.1 VAMP

Two late-acting v-SNARES, VAMP2 (135) and VAMP3/cellubrevin (136), are enriched in GLUT4 vesicles immunoadsorbed from the LDM fractions of either rat fat or 3T3-L1 adipocytes (112, 137). Both VAMP2 and VAMP3 redistribute to the plasma membrane in response to insulin (112,137), and both bind syntaxin 4 *in vitro* (138, 139). Consistent with this, syntaxin 4 mediates the formation of NSF-containing SNARE complexes with both VAMP2 and VAMP3 in *in vitro* membrane reconstitution assays (140, 141), and heterologous expression of the cytoplasmic domain of either VAMP2 or VAMP3 suppresses insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes, perhaps by sequestering syntaxin 4 (142). Similarly, treatment of cells with botulinum neurotoxin D or tetanus toxin, which cleave both VAMP2 and VAMP3, leads to the impairment of GLUT4 translocation and glucose transport (143, 144).

Notwithstanding the apparent redundant behavior of VAMPs 2 and 3, disparate roles have emerged for these two isoforms that appear to relate to compartmental and functional segregation. VAMP2 and VAMP3 appear to define distinct GLUT4 pools, since immunopurified VAMP3-containing GLUT4 vesicles from 3T3-L1 adipocytes do not contain discernible amounts of VAMP2 (137). Consistent with the observation that VAMP3 is involved in constitutively recycling pathways (136), it has been shown that transferrin-HRP ablation of recycling endosomes in 3T3-L1 adipocytes eliminates VAMP3 from the low-density microsomal (LDM) fraction together with a proportion of GLUT4 as well as transferrin receptors, while the residual, non-ablated LDM is markedly enriched in VAMP2-containing GLUT4 vesicles (66). Moreover, this depletion of VAMP3 does not restrict the translocation of the VAMP2-enriched GLUT4 pool in an insulin-dependent manner (67). Similarly in muscle, VAMP2, but not VAMP3, is found in insulin-responsive GLUT4 vesicles (145, 146), while VAMP3 appears to be restricted to endosomal pathways in this tissue (146). Consistent with these findings, insulin-dependent GLUT4 translocation could be rescued by expression of a toxin-resistant form of VAMP2, but not VAMP3, in L6 cells depleted in endogenous VAMPs by tetanus toxin (147). Finally, VAMP3-null mice display normal whole-body glucose metabolism, and normal insulin- and contraction-stimulated glucose transport (148).

It seems then, that VAMP2 plays a central role in trafficking the insulin-responsive GLUT4 pool. Interestingly, VAMP2 is also the operative v-SNARE for other regulated secretory systems such as aquaporin translocation in the kidney duct cells (149), acid secretion from gastric parietal cells (150), insulin secretion from pancreatic islet cells (151) and neurosecretion (135).

At least three classes of accessory molecules have been proposed to play a role in regulating the activity of VAMP2.

5.4.1.1. Prenylated Rab acceptor

Prenylated Rab acceptor (PRA1) was characterized as a molecule that binds both Rab GTPases and VAMP2 in an isoform-specific fashion (152). However, the binding of Rab and VAMP2 to PRA1 is mutually exclusive (153). A role for this protein in GLUT4 trafficking is yet to be established.

VAP-33 (VAMP-associated protein of 33kDa) is an integral membrane VAMP2-interacting protein that has been shown to be broadly expressed across a range of taxa and appears to play a role in exocytosis (154, 155, 156). Manipulating the availability of VAP-33 by overexpression or by microinjection of antibodies to VAP-33 in insulin-responsive cell models modulates GLUT4 exocytosis (157), suggesting that this molecule may be a regulator of VAMP2 in GLUT4 trafficking. However, a definite role for VAP-33 has yet to be established.

5.4.1.3. Pantophysin

Pantophysin is a ubiquitously expressed homologue of neuronal synaptophysin (158). In 3T3-L1 cells it was shown to be upregulated during adipogenesis, was resident as a phosphoprotein on a subpopulation of GLUT4 vesicles basally, and was depleted from vesicles upon insulin treatment (159). Only a fraction of the vesicles containing pantophysin also contain GLUT4, consistent with other roles for this protein in addition to GLUT4 traffic. Nevertheless, pantophysin was found to associate with vesicles containing VAMP2 but not VAMP3 in 3T3-

L1 adipocytes, consistent with a role in the insulin-regulated pathway (159). Synaptophysin, the neuronal homologue of pantophysin, interacts with VAMP and prevents it from binding syntaxin (160). Thus, it is possible that the pantophysin/VAMP interaction may have a regulatory role in SNARE interaction. A specific role for pantophysin in regulating GLUT4 trafficking is yet to emerge, but these studies show it to be an attractive candidate for a role in regulated trafficking.

5.4.2. Syntaxin 4

Amongst the plasma membrane-localized syntaxins in non-neuronal tissues (110), only syntaxin 4 interacts with VAMP2 (138), the v-SNARE implicated in insulin-stimulated trafficking of GLUT4 to the plasma membrane. Syntaxin 4 is expressed in muscle and fat (161, 140, 162), and is upregulated in 3T3-L1 cells upon differentiation into adipocytes (163). In 3T3-L1 adipocytes, a significant proportion of syntaxin 4 is localized to the low-density microsomal fraction, and partially redistributes to the plasma membrane in response to insulin (164). Microinjection, or treatment of permeabilised 3T3-L1 adipocytes with antibodies against syntaxin 4 (139, 164), or with a soluble syntaxin 4 molecule (143, 142) or a syntaxin 4 peptide (144), specifically and significantly reduced insulin-dependent stimulation of GLUT4 translocation and glucose transport, consistent with syntaxin 4 being the major t-SNARE in GLUT4 trafficking.

GLUT4 trafficking is particularly sensitive to the levels of syntaxin 4 in muscle. Heterozygous syntaxin 4 mice suffer a 50% decrease in muscle glucose uptake that parallels a reduction in insulin-stimulated GLUT4 translocation in this tissue, and that renders the animals insulin resistant (6). Interestingly, despite the level of syntaxin 4 being reduced by about 36% in all tissues, insulin responsiveness and glucose uptake in adipose tissue are largely unaffected (6).

Structures for several syntaxins including Vam3p (120), Sso1p (165), syntaxin 7 (118) and syntaxin 1 (166), have been determined. Remarkably, and despite being quite divergent in sequence, subcellular localization and evolution, they are all structurally very similar, consisting of a three-helical N-terminal domain (Habc domain), a C-proximal SNARE motif and a C-terminal transmembrane domain, suggesting that this structural fold is conserved across most, if not all, syntaxins. Syntaxin 4 shares 46% amino acid sequence identity with syntaxin 1 (110) and they share some similar characteristics. VAMP2 is the cognate v-SNARE for both syntaxins, and both bind their respective isoforms of the accessory protein, Munc18, to the exclusion of other SNAREs (139, 167, 168). It is highly likely therefore, that syntaxin 4 adopts a similar fold to, and undergoes a similar itinerary of structural changes as syntaxin 1, dependent on its binding status. Syntaxin 1, either in complex with Munc18a or free, adopts a "closed" conformation, with the SNARE motif stabilized in contact with the N-terminal three-helical domain (119, 166). This fold is similar to that of free, yeast Sso1p (165), suggesting structural conservation that probably extends to syntaxin 4. However, there are slight differences between the bound and unbound forms of syntaxin that may have functional consequences. When syntaxin is bound to Munc18a, the largely helical SNARE motif has bends and other irregularities, and the syntaxin structure does not resemble a canonical four-helix bundle (166). Nevertheless the SNARE motif maintains intimate contact with either the N-terminal Habc domain or with Munc18a across its entire length. Interestingly, the C-terminus of the SNARE motif that is structured and buried in the Munc18 binding pocket (166) is found to be unstructured in free syntaxin (119). This conformational switch of the motif may provide a mechanism for the nucleation of the SNARE complex. The structure adopted by syntaxin 1 in the SNARE complex is different again to both the free and the Munc-liganded form, with the SNARE motif participating in a canonical, parallel, four-helix bundle with the SNARE motifs of VAMP2 and SNAP25 (113, 114). Clearly, syntaxin 1 can adopt a number of stable conformations reflecting its binding status. However, the mechanisms that mediate the transition from one state to another are not yet clear. By virtue of their structural and functional similarity, it is likely that syntaxin 4 adopts a similar itinerary of binding states, although the detail is yet to be elucidated.

In addition to the key syntaxin regulatory protein Munc18c, at least three other molecules are reported to bind to syntaxin 4, possibly as regulators of GLUT4 trafficking. Munc18c and the small GTPase Rab4, which may be involved in tethering vesicles, are dealt with separately herein.

5.4.2.1. Synip

Synip, a 557-residue protein expressed abundantly in skeletal muscle, was isolated from a yeast two-hybrid screen of a 3T3-L1 adipocyte library using syntaxin 4 as bait. This molecule was shown to compete with VAMP2 for syntaxin binding, without affecting the ability of SNAP23 to bind syntaxin. The association of synip with syntaxin 4 is down-regulated by insulin treatment, and insulin-stimulated glucose transport is attenuated by heterologous expression of Synip truncations (169). However, since the original description of this protein, no further detail of its function has emerged for GLUT4 or for any other trafficking process.

5.4.2.2. Cysteine-string protein

Cysteine-string protein (Csp) has been shown to associate with syntaxin 4 at the plasma membrane of adipocytes in an insulin-independent manner (170). While the functional implications of this association are unclear, Csp contains a DnaJ domain, suggesting a possible molecular chaperone role.

5.4.3. SNAP23

The t-SNARE, SNAP23, is a ubiquitously expressed homologue of SNAP25 (109,171). SNAP25 and SNAP23, together with exocytic homologues from a range of taxa (171), share a similar structural topology, with SNARE motifs at the N- and C-terminal ends and a cluster of cysteines centered around residue 83 (172,171) which mediate membrane binding when palmitoylated (173, 174). Sequence identity between SNAP-3 and SNAP25 is

59%, and like SNAP25, SNAP23 interacts *in vitro* with syntaxins 1, 2, 3 and 4 with equal efficiency, as it does with VAMP1 and VAMP2 (109). However, unlike SNAP25 and its cognate neuronal SNAREs, SNAP23 does not markedly potentiate binding of VAMP2 to syntaxin 4 (175), and the formation of SDS-resistant complexes *in vitro* is only observed when SNAP23 is immobilized through its cysteine cluster (176), but not in solution (175, 123). SNAP25 is not thought to be involved in GLUT4 trafficking, as the protein is largely absent from insulin-responsive tissue (176), and the translocation of GLUT4 to the plasma membrane is refractory to the effects of BontA cleavage (144). In contrast, SNAP23 is present in insulin-responsive tissues and, in 3T3-L1 adipocytes at least, at a 3-fold excess over syntaxin 4 (177). The distribution of SNAP23 in 3T3-L1 fibroblasts appears to be perinuclear, with a marked redistribution to the plasma membrane upon differentiation to adipocytes (163). The cysteine-rich region is essential for palmitoylation and plasma membrane localization, and mutations in this region redistribute SNAP23 to the cytoplasm (173, 174).

Interfering with the action of SNAP23, by microinjection of cells with antibody or peptide, inhibits insulin-stimulated GLUT4 translocation (176, 178). Similarly, expression of SNAP23 mutants that bind syntaxin 4, but not VAMP2, also block GLUT4 translocation (179). In contrast, increasing the intracellular concentration of SNAP23 by microinjection of protein or by incubating permeabilised cells with SNAP23, augments the insulin responsiveness of GLUT4 in 3T3-L1 adipocytes (178), suggesting a possible concentration-dependent regulatory role. The binding of SNAP23 to syntaxin 4 is itself blocked by competition with Munc18c in a dosage-dependent manner in heterologously expressing COS cells (168), but in rat adipocytes about 50% of endogenous SNAP23 and syntaxin 4 form stable complexes at the plasma membrane in an insulin-independent fashion (141). Interestingly, complexes consisting of syntaxin 4, SNAP23 and VAMPs could also be isolated from rat adipose cells using an NSF/α-SNAP affinity-binding technique, independent of insulin treatment (141). The mechanisms that favor SNAP23 over Munc18c for binding to syntaxin 4 are unclear, although one report has suggested that binding may be regulated by phosphorylation of SNAP23 by a kinase such as SNAK (SNARE kinase) (180).

5.4.4. Munc18c

The function of the Sec1/Munc18 (SM) family is closely linked to the activity of syntaxin and, in the case of some members at least, the formation and activity of the trimeric SNARE complex. Eukaryotes appear to possess either three or four early-acting SM proteins operating in inter-organelle traffic (vps33, vps45 and sly1), and at least one, late-acting, SM protein that is involved in Golgi-to-plasma membrane traffic (181). Vertebrates possess three closely related isoforms, Munc18a, b and c, which operate at the terminal step of membrane fusion at the plasma membrane. Of these, Munc18a is largely restricted to neuronal tissue (182, 183, 184), whilst Munc18b and Munc18c are more widely distributed (185, 186, 187). Munc18c was originally isolated from a 3T3-L1 adipocyte cDNA library (186) and is the only Munc that binds syntaxin 4 (139, 167). Hence, the apparent role of Munc18c in GLUT4 trafficking has been the subject of some scrutiny.

Several genetic studies across a range of taxa have demonstrated that late-acting SM proteins are essential to exocytosis. Conditional mutants of yeast Sec1 result in the cessation of secretion and arrest of bud growth at the non-permissive temperature (188). Mutations in, or deletion of, unc-18 from *Caenorhabditis elegans* (189), the Drosophila Rop gene (190) and the murine Munc18a gene (191) lead to phenotypes that are characterized by failure of secretion (190), the accumulation of synaptic vesicles (191), and paralysis (189) or death at a late embryonic stage or at birth (190, 191). Although these studies suggest an essential role for SM proteins in fusion, the mechanistic basis for these various phenotypes remain elusive.

Various studies suggest that SM molecules may function in a number of discreet ways, but at all times they exert their activity through SNARE molecules. Indeed, the life cycles of syntaxins and Muncs appear to be closely aligned. Quantitation of the levels of SNARE proteins in 3T3-L1 adipocytes indicates that syntaxin 4, Munc18c and VAMP2 occur in approximately equal, but slightly ascending, concentration (177), suggesting that the stoichiometry of these proteins may somehow be regulated. At least two mechanisms for this regulation present themselves. In mammalian cell lines deficient in endogenous expression of syntaxin 1a or Munc18a, heterologously expressed syntaxin1A accumulates in the Golgi complex rather than at the plasma membrane. This results in disassembly of the Golgi unless rescued by expression of Munc18a and the subsequent delivery of the complex to the plasma membrane (192,193). Conversely, heterologously expressed Munc18c accumulates in the cytoplasmic compartment, but can be depleted from this compartment and mobilized to the plasma membrane upon overexpression of syntaxin 4 (167), a feature in common with Munc18a/syntaxin 1 (194). Curiously, mice that are genetically depleted in syntaxin 4 also exhibit a tight co-ordinate downregulation of Munc18c expression (6). Finally, studies of Tlg2p stability in yeast show that proteasomal degradation of this syntaxin occurs when the Munc homologue Vps45 is genetically ablated (195), providing a further mechanism to maintain a stoichiometric linkage between these two molecules. It appears that Munc18 and syntaxin biosynthesis are tightly linked, and that they may be mutually dependent for correct localization to legitimate sites of vesicle fusion. This co-ordinate regulation of Munc18 with syntaxin may also serve to restrict illegitimate associations of syntaxin in early trafficking compartments, and in the case of syntaxin 4, to acutely regulate its availability in skeletal muscle (6). Consistent with these observations, but as yet untested, is the possibility that the lack of neurotransmission observed in Munc18a-null mice (191), as well as the paralytic phenotype of *unc18*-null *C. elegans*, may be due to a failure in Munc-mediated delivery of syntaxin to sites of secretion.

Notwithstanding this synergistic relationship between Munc and syntaxin, robust experimental evidence suggests that late-acting Muncs play a negative regulatory

role in SNARE complex assembly and vesicle fusion. Tissue-specific overexpression of Munc18c in muscle of a transgenic mouse model inhibits GLUT4 translocation to the transverse tubules without affecting translocation to the sarcolemma (187), whilst overexpression of Munc18c in 3T3-L1 adipocytes inhibits GLUT4 translocation to the plasma membrane (196, 167). Similarly, overexpression of the Drosophila SM protein, Rop, leads to a decrease in neurotransmitter release (197). Conversely, Drosophila with mutations in syntaxin that eliminate binding to Rop display increased neurotransmitter release, suggesting that Rop inhibits neurosecretion through its interaction with syntaxin (198). Finally, microinjection of 3T3-L1 adipocytes with antibodies to Munc18c increases plasma membrane association of GLUT4 under basal conditions, without affecting the insulin-stimulated translocation of GLUT4 (199), and administration of Munc18a antibodies to permeabilised HIT-T15 cells increases insulin secretion without affecting stimulus-coupled secretion of insulin (200). These data suggest that Munc18 exerts an inhibitory tone on vesicle fusion that can be relieved by immunosequestration of Munc18 under basal conditions and is independent of physiological stimuli.

These data beg the question as to the molecular mechanism that underlies this inhibitory effect. Given that Munc18 seems to operate through its interaction with syntaxin, the mechanism involved might be as simple as conformational constriction of syntaxin, regulating the formation of SNARE complexes. Evidence favoring this explanation has come from several sources. *In vitro* protein-protein interaction studies have demonstrated that Munc18c occludes syntaxin 4, thus preventing VAMP2 or SNAP23 binding to it (139, 167, 168). *In vivo* studies have shown that the neuronal homologue, Munc18a, forms complexes containing syntaxin1a but not SNAP25 or VAMP2, preventing syntaxin 1a from forming SNARE complexes (201). Finally, the three-dimensional crystal structure of the Munc18a/syntaxin 1a complex shows that syntaxin is bound in a "closed" conformation that precludes SNARE complex formation (166). A robust mechanism for the alleviation of this negative regulatory role is yet to emerge, and yet some interesting observations have been made in this area. Insulin stimulation disrupts the association between Munc18c and syntaxin 4 (167). This disruption may be mediated through a direct phosphorylation event, as phosphorylation of Munc18a has been shown to disrupt its association with syntaxin 1a (202, 203). However, a mutagenesis study of the equivalent phosphorylation site in Munc18c failed to detect any difference in behavior between wild-type and mutant Munc18c with respect to syntaxin 4 binding and glucose transport (199). Alternatively, alleviation of Munc18c's inhibitory effects may be mediated through an effector molecule. In support of this possibility, microinjection of a Munc18c peptide into 3T3-L1 adipocytes results in the inhibition of insulin-stimulated plasma membrane fusion of GLUT4 vesicles and accumulation of vesicles just below the plasma membrane (204), indicative that trafficking, but not fusion, has occurred. This peptide maps to a non-syntaxin binding region of Munc18c and thus may represent the binding site for an as yet unidentified effector.

While attractive, this negative functional role for Munc18c may yet prove too simple, as interesting observations have been made with yeast, as well as early-acting SM proteins, suggesting additional roles for these proteins. In yeast, the association between SM protein Sec1p and the t-SNARE Sso2p is weak (205). Rather, Sec1p is reported to interact with the pre-assembled trimeric SNARE complex at sites of secretion. Sec1 possibly shields the SNARE complex from Sec18p-mediated disassembly or perhaps stimulates fusion, but in any event it acts at a stage intermediate between SNARE complex assembly and NSF/sec18-mediated disassembly (121). It is possible that endomembrane SM molecules may facilitate SNARE complex assembly. In both yeast and mammals, early-acting SM molecules, Vps45p and Sly1p, bind to their respective t-SNARES, Tlg2/syntaxin 16 and Sed5p/syntaxin 18, via short peptide motifs N-terminal to the Habc regulatory domain of the t-SNARE (206, 207, 208), whilst the remainder of the t-SNARE molecule, at least in the case of Tlg2p/syntaxin 16, maintains an open conformation (205,206). The crystal structure of Sly1p in complex with the N-terminal, 45 residue, peptide from Sed5p indicates that the peptide forms two small helices that bind to a novel location on domain 1 of Sly1p, on the opposite side to the syntaxin 1 interaction site with Munc18a (209). This mode of binding appears to allow SNARE complex assembly to take place on the syntaxin while it is still bound to Sly1p, an association that may impose specificity on SNARE complex formation (208). These observations are in contrast to the behavior of Munc18a which binds syntaxin1a in a "closed" conformation (119, 201) and utilizes multiple contact sites across the whole of syntaxin (166). Unfortunately, structural information regarding the binding conformation of syntaxin 4 and Munc18c is yet to emerge.

The regulatory effect of Munc18c on insulin-responsive GLUT4 translocation may therefore act at several points, ranging from co-translocation with syntaxin 4 to the plasma membrane and preventing non-specific SNARE pairings, to regulating the availability of syntaxin 4 for SNARE-complex formation in response to physiological stimuli. The proposition that Munc18c cycles through a stage of binding SNARE complexes, as is the case with yeast Sec1p (121), cannot be excluded.

5.4.5. Rab GTPases

The Rab family of small GTPases are key regulators of membrane traffic. Up to 60 Rab genes have been recognized in the human genome (181), and specific isoforms appear to be localized to discreet membrane compartments. Rabs function as molecular switches, naturally cycling between the GDP-bound "off" state and the GTP-bound "on" state at intrinsically slow rates. These slow rates of hydrolysis and nucleotide-exchange of Rab proteins may be dramatically enhanced or suppressed by nucleotide exchange factors, GDP-dissociation inhibitors and GTPase activation proteins. These regulatory molecules offer further opportunities for temporal control of Rab-mediated processes by hormones such as insulin, and other regulatory factors.

By virtue of their organelle distribution, Rabs confer a degree of spatial and temporal specificity to

membrane trafficking events (210). They have been implicated in cargo selection (211, 212), vesicle motility (213, 214), vesicle tethering (134, 215) and membrane fusion (216, 217). This wide range of GTP-Rab functions is mediated by an extraordinary diversity of effector molecules.

A major role for Rab proteins is in linking the vesicle to the opposing membrane via tethering proteins (210). For example, Rab5 binding to EEA1 has been implicated in linking endocytic vesicles to early endosomes (218, 219). The reported *in vivo* binding of Rab4 and syntaxin 4 (220) may represent a tethering interaction with physiological relevance to GLUT4 trafficking. This interaction is dependent on Rab4 being in the GTP-bound state (220), and the activation of Rab4 is sensitive to insulin-stimulated guanine nucleotide exchange on the GTPase in a wortmannin-dependent fashion (221).

Rabs and Rab effectors may also link with the SNARE machinery through the Sec1/Munc18 proteins. An initial indication was the discovery of yeast Sly1 as a genetic suppressor of the loss of Ypt1 function (222). Currently, the following SM proteins have been reported as constituent members of Rab effector complexes: Vps45 with Rabenosyn-5 at the endosome (223), Vps33 at the vacuole (224, 225) and Vps45 with Vac1 (226). These effector complexes may play a role in the regulation of SNARE complex formation (227), in addition to regulating Rab function by modulating their GTP status (224).

To date, two Rab family members, Rab4 and Rab11 have been found to colocalize on membranes with GLUT4 and to re-distribute in an insulin-sensitive manner. Rab11 is predominantly localized to the perinuclear recycling endosomes (228). In cardiac muscle it was found to localize with GLUT4, both in this compartment as well as to non-endosomal GLUT4 storage vesicles (229). In response to insulin, Rab11 is reported to relocate to the plasma membrane, as well as to redistribute from the endosomal pool to the GLUT4 storage vesicles, suggesting a role in trafficking GLUT4 to the glucose storage vesicle (229). Rabphilin-11/Rab11BP is an effector of Rab11 function (230, 231), which binds to a domain bounded by residues 334 to 504 of the GTPase (230). Expression of this fragment in adipocytes (73) caused the redistribution of GLUT4 from a TfR-negative compartment to one which is TfR-positive, and inhibited insulin-stimulated translocation of GLUT4 to the plasma membrane (73). These data suggest a key role for Rab11 in withdrawing a proportion of GLUT4 from the general TfR-positive recycling pool to the specialized GLUT4 storage vesicle pool (73).

Rab4 has been suggested to control traffic between the plasma membrane and the early endosomal compartment (232) by regulating the formation of recycling vesicles (233). In muscle and adipocytes, Rab4 has been found to colocalize with GLUT4 on immunopurified vesicles (234, 235) and, to redistribute to the cytosol in synchrony with the translocation of GLUT4 to the plasma membrane upon insulin stimulation (234). This redistribution can be abolished by wortmannin, suggesting involvement of the PI 3-kinase pathway (221). Interference assays have demonstrated that adipocytes that have been electroporated with a Rab4 C-terminal peptide (236), or that heterologously overexpress wild-type or hydrolysis-deficient mutants of Rab4 (237), display decreased GLUT4 translocation and glucose uptake in response to insulin. Thus Rab4 appears to play a role in GLUT4 translocation by an undefined mechanism. Interestingly, insulin has been shown to stimulate the geranylgeranylation of Rab4, a prerequisite for its membrane localization and GTP loading/activation (238), providing an additional means for hormone regulation.

6. PERSPECTIVES AND CONCLUSIONS

Significant inroads have been made in cataloguing the molecular repertoire and understanding the processes involved in GLUT4 vesicle translocation and fusion. Much of this information has been derived by analogy with other regulated trafficking events, including neurotransmitter trafficking in neuronal cells and understanding the basis of yeast secretion mutants. It has been established that many of the underlying principles governing regulated membrane trafficking and fusion have been conserved through evolution. Vesicle fusion is directed by the formation of a SNARE complex between the fusing vesicle SNARE protein and its target membrane SNAREs to enable fusion. In the case of GLUT4 vesicle fusion, VAMP2 on the vesicle forms a SNARE complex with syntaxin 4 and SNAP23 on the plasma membrane. These SNAREs are similar, though not identical, to SNAREs for neuronal vesicle trafficking. Regulation of the fusion process involves accessory proteins that include munc18c, Rabs and several less well characterized proteins such as synip, pantophysin, VAP-33, but the detail of this regulation remains poorly understood. Data to date suggest that these proteins are involved in regulating the availability and/or tethering SNAREs for fusion. How these processes are linked to the initial insulin signaling events remains an open question as are the identities of the protein(s) targeted, but further development in this area can be expected over coming years. Additionally, it is possible that subcellular compartmentalization of the signaling machinery may play a significant role. The initial stages of insulin signaling involves a well established phosphorylation cascade, however data concerning the phosphorylation of the SNARE proteins involved in trafficking GLUT4 have provided little evidence to support this mode of regulation of the fusion process. The processes involved in endocytic trafficking of GLUT4 are even less well understood, however it is possible that the biogenesis of the insulin-responsive GLUT4 compartment, and understanding of the SNAREs directing traffic to this compartment, may offer new targets for therapies directed at ameliorating insulin resistance. Further studies of the function and regulation of these SNARE proteins will provide basic knowledge that should delineate this process and contribute to a better understanding of the mechanisms underlying insulin resistance and type II diabetes.

7. ACKNOWLEDGEMENTS

We thank Dr Morry Frenkel and Ms Danila Durante for helpful comments and discussion.

8. REFERENCES

1. Charron M. J., F. C. Brosius 3rd, S. L. Alper & H. F. Lodish: A glucose transport protein expressed predominately in insulin- responsive tissues. *Proc Natl Acad Sci U S A* 86, 2535-9 (1989)
2. James D. E., M. Strube & M. Mueckler: Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* 338, 83-7 (1989)
3. James D. E., R. Brown, J. Navarro & P. F. Pilch: Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature* 333, 183-5 (1988)
4. Birnbaum M. J.: Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* 57, 305-15 (1989)
5. Maier V. H., D. R. Melvin, C. A. Lister, H. Chapman, G. W. Gould & G. J. Murphy: v- and t-SNARE protein expression in models of insulin resistance: normalization of glycemia by rosiglitazone treatment corrects overexpression of cellubrevin, vesicle-associated membrane protein-2, and syntaxin 4 in skeletal muscle of Zucker diabetic fatty rats. *Diabetes* 49, 618-25 (2000)
6. Yang C., K. J. Coker, J. K. Kim, S. Mora, D. C. Thurmond, A. C. Davis, B. Yang, R. A. Williamson, G. I. Shulman & J. E. Pessin: Syntaxin 4 heterozygous knockout mice develop muscle insulin resistance. *J Clin Invest* 107, 1311-8 (2001)
7. White M. F. & C. R. Kahn: The insulin signaling system. *J Biol Chem* 269, 1-4 (1994)
8. Ullrich A., J. R. Bell, E. Y. Chen, R. Herrera, L. M. Petruzzelli, T. J. Dull, A. Gray, L. Coussens, Y. C. Liao, M. Tsubokawa & a. l. et: Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 313, 756-61 (1985)
9. Ebina Y., M. Edery, L. Ellis, D. Standring, J. Beaudoin, R. A. Roth & W. J. Rutter: Expression of a functional human insulin receptor from a cloned cDNA in Chinese hamster ovary cells. *Proc Natl Acad Sci U S A* 82, 8014-8 (1985)
10. Ebina Y., L. Ellis, K. Jarnagin, M. Edery, L. Graf, E. Clauser, J. H. Ou, F. Masiarz, Y. W. Kan, I. D. Goldfine & a. l. et: The human insulin receptor cDNA: the structural basis for hormone- activated transmembrane signalling. *Cell* 40, 747-58 (1985)
11. Hubbard S. R., L. Wei, L. Ellis & W. A. Hendrickson: Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature* 372, 746-54 (1994)
12. Backer J. M., M. G. Myers Jr, S. E. Shoelson, D. J. Chin, X. J. Sun, M. Miralpeix, P. Hu, B. Margolis, E. Y. Skolnik, J. Schlessinger & a. l. et: Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO J* 11, 3469-79 (1992)
13. Arcaro A. & M. P. Wymann: Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* 296 (Pt 2), 297-301 (1993)
14. Kanai F., K. Ito, M. Todaka, H. Hayashi, S. Kamohara, K. Ishii, T. Okada, O. Hazeki, M. Ui & Y. Ebina: Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase. *Biochem Biophys Res Commun* 195, 762-8 (1993)
15. Okada T., Y. Kawano, T. Sakakibara, O. Hazeki & M. Ui: Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. *J Biol Chem* 269, 3568-73 (1994)
16. Yeh J. I., E. A. Gulve, L. Rameh & M. J. Birnbaum: The effects of wortmannin on rat skeletal muscle. Dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *J Biol Chem* 270, 2107-11 (1995)
17. Lund S., G. D. Holman, O. Schmitz & O. Pedersen: Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A* 92, 5817-21 (1995)
18. Le Marchand-Brustel Y., N. Gautier, M. Cormont & E. Van Obberghen: Wortmannin inhibits the action of insulin but not that of okadaic acid in skeletal muscle: comparison with fat cells. *Endocrinology* 136, 3564-70 (1995)
19. Jiang T., G. Sweeney, M. T. Rudolf, A. Klip, A. Traynor-Kaplan & R. Y. Tsien: Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* 273, 11017-24 (1998)
20. Alessi D. R., S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese & P. Cohen: Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7, 261-9 (1997)
21. Le Good J. A., W. H. Ziegler, D. B. Parekh, D. R. Alessi, P. Cohen & P. J. Parker: Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281, 2042-5 (1998)
22. Standaert M. L., G. Bandyopadhyay, Y. Kanoh, M. P. Sajan & R. V. Farese: Insulin and PIP3 activate PKC-zeta by mechanisms that are both dependent and independent of phosphorylation of activation loop (T410) and autophosphorylation (T560) sites. *Biochemistry* 40, 249-55 (2001)
23. Standaert M. L., G. Bandyopadhyay, L. Perez, D. Price, L. Galloway, A. Poklepovic, M. P. Sajan, V. Cenni, A. Sirri, J. Moscat, A. Toker & R. V. Farese: Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274, 25308-16 (1999)
24. Filippa N., C. L. Sable, B. A. Hemmings & E. Van Obberghen: Effect of phosphoinositide-dependent kinase 1 on protein kinase B translocation and its subsequent activation. *Mol Cell Biol* 20, 5712-21 (2000)
25. Altomare D. A., G. E. Lyons, Y. Mitsuuchi, J. Q. Cheng & J. R. Testa: Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene* 16, 2407-11 (1998)
26. Hill M. M., S. F. Clark, D. F. Tucker, M. J. Birnbaum, D. E. James & S. L. Macaulay: A role for protein kinase Bbeta/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. *Mol Cell Biol* 19, 7771-81 (1999)
27. Walker K. S., M. Deak, A. Paterson, K. Hudson, P. Cohen & D. R. Alessi: Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro:

comparison with protein kinase B alpha. *Biochem J* 331 (Pt 1), 299-308 (1998)
28. Mitsuuchi Y., S. W. Johnson, S. Moonblatt & J. R. Testa: Translocation and activation of AKT2 in response to stimulation by insulin. *J Cell Biochem* 70, 433-41 (1998)
29. Calera M. R., C. Martinez, H. Liu, A. K. Jack, M. J. Birnbaum & P. F. Pilch: Insulin increases the association of Akt-2 with Glut4-containing vesicles. *J Biol Chem* 273, 7201-4 (1998)
30. Kupriyanova T. A. & K. V. Kandror: Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *J Biol Chem* 274, 1458-64 (1999)
31. Ducluzeau P. H., L. M. Fletcher, G. I. Welsh & J. M. Tavare: Functional consequence of targeting protein kinase B/Akt to GLUT4 vesicles. *J Cell Sci* 115, 2857-66 (2002)
32. Tanti J. F., S. Grillo, T. Gremeaux, P. J. Coffer, E. Van Obberghen & Y. Le Marchand-Brustel: Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes. *Endocrinology* 138, 2005-10 (1997)
33. Bandyopadhyay G., M. L. Standaert, M. P. Sajan, L. M. Karnitz, L. Cong, M. J. Quon & R. V. Farese: Dependence of insulin-stimulated glucose transporter 4 translocation on 3-phosphoinositide-dependent protein kinase-1 and its target threonine- 410 in the activation loop of protein kinase C-zeta. *Mol Endocrinol* 13, 1766-72 (1999)
34. Cho H., J. L. Thorvaldsen, Q. Chu, F. Feng & M. J. Birnbaum: Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276, 38349-52 (2001)
35. Cho H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw 3rd, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman & M. J. Birnbaum: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292, 1728-31 (2001)
36. Bandyopadhyay G., M. P. Sajan, Y. Kanoh, M. L. Standaert, M. J. Quon, R. Lea-Currie, A. Sen & R. V. Farese: PKC-zeta mediates insulin effects on glucose transport in cultured preadipocyte-derived human adipocytes. *J Clin Endocrinol Metab* 87, 716-23 (2002)
37. Etgen G. J., K. M. Valasek, C. L. Broderick & A. R. Miller: In vivo adenoviral delivery of recombinant human protein kinase C-zeta stimulates glucose transport activity in rat skeletal muscle. *J Biol Chem* 274, 22139-42 (1999)
38. Kotani K., W. Ogawa, M. Matsumoto, T. Kitamura, H. Sakaue, Y. Hino, K. Miyake, W. Sano, K. Akimoto, S. Ohno & M. Kasuga: Requirement of atypical protein kinase clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol* 18, 6971-82 (1998)
39. Vollenweider P., B. Menard & P. Nicod: Insulin resistance, defective insulin receptor substrate 2-associated phosphatidylinositol-3' kinase activation, and impaired atypical protein kinase C (zeta/lambda) activation in myotubes from obese patients with impaired glucose tolerance. *Diabetes* 51, 1052-9 (2002)
40. Tremblay F., C. Lavigne, H. Jacques & A. Marette: Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (zeta/lambda) activities. *Diabetes* 50, 1901-10 (2001)
41. Hodgkinson C. P., E. M. Sale & G. J. Sale: Characterization of PDK2 activity against protein kinase B gamma. *Biochemistry* 41, 10351-9 (2002)
42. Isakoff S. J., C. Taha, E. Rose, J. Marcusohn, A. Klip & E. Y. Skolnik: The inability of phosphatidylinositol 3-kinase activation to stimulate GLUT4 translocation indicates additional signaling pathways are required for insulin-stimulated glucose uptake. *Proc Natl Acad Sci U S A* 92, 10247-51 (1995)
43. Frevert E. U. & B. B. Kahn: Differential effects of constitutively active phosphatidylinositol 3- kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol Cell Biol* 17, 190-8 (1997)
44. Liu J., A. Kimura, C. A. Baumann & A. R. Saltiel: APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. *Mol Cell Biol* 22, 3599-609 (2002)
45. Baumann C. A., V. Ribon, M. Kanzaki, D. C. Thurmond, S. Mora, S. Shigematsu, P. E. Bickel, J. E. Pessin & A. R. Saltiel: CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* 407, 202-7 (2000)
46. Chiang S. H., C. A. Baumann, M. Kanzaki, D. C. Thurmond, R. T. Watson, C. L. Neudauer, I. G. Macara, J. E. Pessin & A. R. Saltiel: Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* 410, 944-8 (2001)
47. Saltiel A. R. & J. E. Pessin: Insulin signaling pathways in time and space. *Trends Cell Biol* 12, 65-71 (2002)
48. Jiang Z. Y., A. Chawla, A. Bose, M. Way & M. P. Czech: A phosphatidylinositol 3-kinase-independent insulin signaling pathway to N-WASP/Arp2/3/F-actin required for GLUT4 glucose transporter recycling. *J Biol Chem* 277, 509-15 (2002)
49. Kanzaki M., R. T. Watson, J. C. Hou, M. Stamnes, A. R. Saltiel & J. E. Pessin: Small GTP-binding Protein TC10 Differentially Regulates Two Distinct Populations of Filamentous Actin in 3T3L1 Adipocytes. *Mol Biol Cell* 13, 2334-46 (2002)
50. Kanzaki M. & J. E. Pessin: Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. *J Biol Chem* 276, 42436-44 (2001)
51. Chang L., R. D. Adams & A. R. Saltiel: The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. *Proc Natl Acad Sci U S A* 99, 12835-40 (2002)
52. Tian L., D. L. Nelson & D. M. Stewart: Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J Biol Chem* 275, 7854-61 (2000)
53. Kanzaki M. & J. E. Pessin: Caveolin-associated filamentous actin (Cav-actin) defines a novel F- actin structure in adipocytes. *J Biol Chem* 277, 25867-9 (2002)
54. Parton R. G., J. C. Molero, M. Floetenmeyer, K. M. Green & D. E. James: Characterization of a distinct plasma membrane macrodomain in differentiated adipocytes. *J Biol Chem* 2002)
55. Sweeney G., R. Somwar, T. Ramlal, A. Volchuk, A. Ueyama & A. Klip: An inhibitor of p38 mitogen-activated protein kinase prevents insulin- stimulated glucose transport but not glucose transporter translocation in 3T3-

L1 adipocytes and L6 myotubes. *J Biol Chem* 274, 10071-8 (1999)
56. Hausdorff S. F., D. C. Fingar, K. Morioka, L. A. Garza, E. L. Whiteman, S. A. Summers & M. J. Birnbaum: Identification of wortmannin-sensitive targets in 3T3-L1 adipocytes. DissociationoOf insulin-stimulated glucose uptake and glut4 translocation. *J Biol Chem* 274, 24677-84 (1999)
57. Somwar R., W. Niu, D. Y. Kim, G. Sweeney, V. K. Randhawa, C. Huang, T. Ramlal & A. Klip: Differential effects of phosphatidylinositol 3-kinase inhibition on intracellular signals regulating GLUT4 translocation and glucose transport. *J Biol Chem* 276, 46079-87 (2001)
58. Malide D., G. Ramm, S. W. Cushman & J. W. Slot: Immunoelectron microscopic evidence that GLUT4 translocation explains the stimulation of glucose transport in isolated rat white adipose cells. *J Cell Sci* 113 Pt 23, 4203-10 (2000)
59. Slot J. W., H. J. Geuze, S. Gigengack, D. E. James & G. E. Lienhard: Translocation of the glucose transporter GLUT4 in cardiac myocytes of the rat. *Proc Natl Acad Sci U S A* 88, 7815-9 (1991)
60. Ploug T., B. van Deurs, H. Ai, S. W. Cushman & E. Ralston: Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J Cell Biol* 142, 1429-46 (1998)
61. Ramm G., J. W. Slot, D. E. James & W. Stoorvogel: Insulin recruits GLUT4 from specialized VAMP2-carrying vesicles as well as from the dynamic endosomal/trans-Golgi network in rat adipocytes. *Mol Biol Cell* 11, 4079-91 (2000)
62. Smith R. M., M. J. Charron, N. Shah, H. F. Lodish & L. Jarett: Immunoelectron microscopic demonstration of insulin-stimulated translocation of glucose transporters to the plasma membrane of isolated rat adipocytes and masking of the carboxyl-terminal epitope of intracellular GLUT4. *Proc Natl Acad Sci U S A* 88, 6893-7 (1991)
63. Munoz P., M. Rosemblatt, X. Testar, M. Palacin, G. Thoidis, P. F. Pilch & A. Zorzano: The T-tubule is a cell-surface target for insulin-regulated recycling of membrane proteins in skeletal muscle. *Biochem J* 312 (Pt 2), 393-400 (1995)
64. Simpson I. A., D. R. Yver, P. J. Hissin, L. J. Wardzala, E. Karnieli, L. B. Salans & S. W. Cushman: Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. *Biochim Biophys Acta* 763, 393-407 (1983)
65. Livingstone C., D. E. James, J. E. Rice, D. Hanpeter & G. W. Gould: Compartment ablation analysis of the insulin-responsive glucose transporter (GLUT4) in 3T3-L1 adipocytes. *Biochem J* 315 (Pt 2), 487-95 (1996)
66. Martin S., J. Tellam, C. Livingstone, J. W. Slot, G. W. Gould & D. E. James: The glucose transporter (GLUT-4) and vesicle-associated membrane protein-2 (VAMP-2) are segregated from recycling endosomes in insulin- sensitive cells. *J Cell Biol* 134, 625-35 (1996)
67. Martin L. B., A. Shewan, C. A. Millar, G. W. Gould & D. E. James: Vesicle-associated membrane protein 2 plays a specific role in the insulin-dependent trafficking of the facilitative glucose transporter GLUT4 in 3T3-L1 adipocytes. *J Biol Chem* 273, 1444-52 (1998)
68. Kupriyanova T. A., V. Kandror & K. V. Kandror: Isolation and characterization of the two major intracellular Glut4 storage compartments. *J Biol Chem* 277, 9133-8 (2002)
69. Kandror K. V.: Insulin regulation of protein traffic in rat adipose cells. *J Biol Chem* 274, 25210-7 (1999)
70. Bryant N. J., R. Govers & D. E. James: Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol* 3, 267-77 (2002)
71. Martin S., G. Ramm, C. T. Lyttle, T. Meerloo, W. Stoorvogel & D. E. James: Biogenesis of insulin-responsive GLUT4 vesicles is independent of brefeldin A-sensitive trafficking. *Traffic* 1, 652-60 (2000)
72. Malide D., N. K. Dwyer, E. J. Blanchette-Mackie & S. W. Cushman: Immunocytochemical evidence that GLUT4 resides in a specialized translocation post-endosomal VAMP2-positive compartment in rat adipose cells in the absence of insulin. *J Histochem Cytochem* 45, 1083-96 (1997)
73. Zeigerer A., M. A. Lampson, O. Karylowski, D. D. Sabatini, M. Adesnik, M. Ren & T. E. McGraw: GLUT4 Retention in Adipocytes Requires Two Intracellular Insulin-regulated Transport Steps. *Mol Biol Cell* 13, 2421-35 (2002)
74. Jiang H., J. Li, E. B. Katz & M. J. Charron: GLUT4 ablation in mice results in redistribution of IRAP to the plasma membrane. *Biochem Biophys Res Commun* 284, 519-25 (2001)
75. Malide D., J. F. St-Denis, S. R. Keller & S. W. Cushman: Vp165 and GLUT4 share similar vesicle pools along their trafficking pathways in rat adipose cells. *FEBS Lett* 409, 461-8 (1997)
76. Garza L. A. & M. J. Birnbaum: Insulin-responsive aminopeptidase trafficking in 3T3-L1 adipocytes. *J Biol Chem* 275, 2560-7 (2000)
77. Keller S. R., A. C. Davis & K. B. Clairmont: Mice deficient in the insulin-regulated membrane aminopeptidase show substantial decreases in glucose transporter GLUT4 levels but maintain normal glucose homeostasis. *J Biol Chem* 277, 17677-86 (2002)
78. Tortorella L. L. & P. F. Pilch : C2C12 myocytes lack an insulin-responsive vesicular compartment despite dexamethasone-induced GLUT4 expression. *Am J Physiol Endocrinol Metab* 283, E514-24 (2002)
79. Kao A. W., B. P. Ceresa, S. R. Santeler & J. E. Pessin: Expression of a dominant interfering dynamin mutant in 3T3L1 adipocytes inhibits GLUT4 endocytosis without affecting insulin signaling. *J Biol Chem* 273, 25450-7 (1998)
80. Volchuk A., S. Narine, L. J. Foster, D. Grabs, P. De Camilli & A. Klip: Perturbation of dynamin II with an amphiphysin SH3 domain increases GLUT4 glucose transporters at the plasma membrane in 3T3-L1 adipocytes. Dynamin II participates in GLUT4 endocytosis. *J Biol Chem* 273, 8169-76 (1998)
81. Al-Hasani H., C. S. Hinck & S. W. Cushman: Endocytosis of the glucose transporter GLUT4 is mediated by the GTPase dynamin. *J Biol Chem* 273, 17504-10 (1998)
82. Karlsson M., H. Thorn, S. Parpal, P. Stralfors & J. Gustavsson: Insulin induces translocation of glucose transporter GLUT4 to plasma membrane caveolae in adipocytes. *FASEB J* 16, 249-51 (2002)
83. Ros-Baro A., C. Lopez-Iglesias, S. Peiro, D. Bellido, M. Palacin, A. Zorzano & M. Camps: Lipid rafts are

required for GLUT4 internalization in adipose cells. *Proc Natl Acad Sci U S A* 98, 12050-5 (2001)84. Foster L. J., D. Li, V. K. Randhawa & A. Klip: Insulin accelerates inter-endosomal GLUT4 traffic via phosphatidylinositol 3-kinase and protein kinase B. *J Biol Chem* 276, 44212-21 (2001)

85. Lalioti V., S. Vergarajauregui & I. V. Sandoval: Targeting motifs in GLUT4 (review). *Mol Membr Biol* 18, 257-64 (2001)

86. Shigematsu S., A. H. Khan, M. Kanzaki & J. E. Pessin: Intracellular insulin-responsive glucose transporter (GLUT4) distribution but not insulin-stimulated GLUT4 exocytosis and recycling are microtubule dependent. *Mol Endocrinol* 16, 1060-8 (2002)

87. Molero J. C., J. P. Whitehead, T. Meerloo & D. E. James: Nocodazole inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes via a microtubule-independent mechanism. *J Biol Chem* 276, 43829-35 (2001)

88. Huang J., T. Imamura & J. M. Olefsky: Insulin can regulate GLUT4 internalization by signaling to Rab5 and the motor protein dynein. *Proc Natl Acad Sci U S A* 98, 13084-9 (2001)

89. Fletcher L. M., G. I. Welsh, P. B. Oatey & J. M. Tavare: Role for the microtubule cytoskeleton in GLUT4 vesicle trafficking and in the regulation of insulin-stimulated glucose uptake. *Biochem J* 352 Pt 2, 267-76 (2000)

90. Omata W., H. Shibata, L. Li, K. Takata & I. Kojima: Actin filaments play a critical role in insulin-induced exocytotic recruitment but not in endocytosis of GLUT4 in isolated rat adipocytes. *Biochem J* 346 Pt 2, 321-8 (2000)

91. Patki V., J. Buxton, A. Chawla, L. Lifshitz, K. Fogarty, W. Carrington, R. Tuft & S. Corvera: Insulin action on GLUT4 traffic visualized in single 3T3-l1 adipocytes by using ultra-fast microscopy. *Mol Biol Cell* 12, 129-41 (2001)

92. Rothman J. E., R. L. Miller & L. J. Urbani: Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. *J Cell Biol* 99, 260-71 (1984)

93. Balch W. E., W. G. Dunphy, W. A. Braell & J. E. Rothman: Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N- acetylglucosamine. *Cell* 39, 405-16 (1984)

94. Braell W. A., W. E. Balch, D. C. Dobbertin & J. E. Rothman: The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of cisternae. *Cell* 39, 511-24 (1984)

95. Balch W. E., B. S. Glick & J. E. Rothman: Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* 39 , 525-36 (1984)

96. Block M. R., B. S. Glick, C. A. Wilcox, F. T. Wieland & J. E. Rothman: Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc Natl Acad Sci U S A* 85, 7852-6 (1988)

97. Wilson D. W., C. A. Wilcox, G. C. Flynn, E. Chen, W. J. Kuang, W. J. Henzel, M. R. Block, A. Ullrich & J. E. Rothman: A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature* 339, 355-9 (1989)

98. Novick P., C. Field & R. Schekman: Identification of 23 complementation groups required for post- translational events in the yeast secretory pathway. *Cell* 21, 205-15 (1980)

99. Clary D. O., I. C. Griff & J. E. Rothman: SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell* 61, 709-21 (1990)

100. Griff I. C., R. Schekman, J. E. Rothman & C. A. Kaiser: The yeast SEC17 gene product is functionally equivalent to mammalian alpha-SNAP protein. *J Biol Chem* 267, 12106-15 (1992)

101. Kaiser C. A. & R. Schekman: Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell* 61, 723-33 (1990)

102. Weidman P. J., P. Melancon, M. R. Block & J. E. Rothman: Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J Cell Biol* 108, 1589-96 (1989)

103. Wilson D. W., S. W. Whiteheart, M. Wiedmann, M. Brunner & J. E. Rothman: A multisubunit particle implicated in membrane fusion. *J Cell Biol* 117, 531-8 (1992)

104. Sollner T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst & J. E. Rothman: SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318-24 (1993)

105. Bennett M. K., N. Calakos & R. H. Scheller: Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* 257, 255-9 (1992)

106. Baumert M., P. R. Maycox, F. Navone, P. De Camilli & R. Jahn: Synaptobrevin: an integral membrane protein of 18,000 daltons present in small synaptic vesicles of rat brain. *EMBO J* 8, 379-84 (1989)

107. Sudhof T. C., M. Baumert, M. S. Perin & R. Jahn: A synaptic vesicle membrane protein is conserved from mammals to Drosophila. *Neuron* 2, 1475-81 (1989)

108. Oyler G. A., G. A. Higgins, R. A. Hart, E. Battenberg, M. Billingsley, F. E. Bloom & M. C. Wilson: The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J Cell Biol* 109, 3039-52 (1989)

109. Ravichandran V., A. Chawla & P. A. Roche: Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J Biol Chem* 271, 13300-3 (1996)

110. Bennett M. K., J. E. Garcia-Arraras, L. A. Elferink, K. Peterson, A. M. Fleming, C. D. Hazuka & R. H. Scheller: The syntaxin family of vesicular transport receptors. *Cell* 74, 863-73 (1993)

111. Hay J. C.: SNARE complex structure and function. *Exp Cell Res* 271, 10-21 (2001)

112. Cain C. C., W. S. Trimble & G. E. Lienhard: Members of the VAMP family of synaptic vesicle proteins are components of glucose transporter-containing vesicles from rat adipocytes. *J Biol Chem* 267, 11681-4 (1992)

113. Sutton R. B., D. Fasshauer, R. Jahn & A. T. Brunger: Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature* 395, 347-53 (1998)

114. Poirier M. A., W. Xiao, J. C. Macosko, C. Chan, Y. K. Shin & M. K. Bennett: The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nat Struct Biol* 5, 765-9 (1998)
115. Antonin W., D. Fasshauer, S. Becker, R. Jahn & T. R. Schneider: Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat Struct Biol* 9, 107-11 (2002)
116. Ungermann C., G. F. von Mollard, O. N. Jensen, N. Margolis, T. H. Stevens & W. Wickner: Three v-SNAREs and two t-SNAREs, present in a pentameric cis-SNARE complex on isolated vacuoles, are essential for homotypic fusion. *J Cell Biol* 145, 1435-42 (1999)
117. Fernandez I., J. Ubach, I. Dulubova, X. Zhang, T. C. Sudhof & J. Rizo: Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell* 94, 841-9 (1998)
118. Antonin W., I. Dulubova, D. Arac, S. Pabst, J. Plitzner, J. Rizo & R. Jahn: The N-terminal Domains of Syntaxin 7 and vti1b Form Three-helix Bundles That Differ in Their Ability to Regulate SNARE Complex Assembly. *J Biol Chem* 277, 36449-56 (2002)
119. Dulubova I., S. Sugita, S. Hill, M. Hosaka, I. Fernandez, T. C. Sudhof & J. Rizo: A conformational switch in syntaxin during exocytosis: role of munc18 . *EMBO J* 18, 4372-82 (1999)
120. Dulubova I., T. Yamaguchi, Y. Wang, T. C. Sudhof & J. Rizo: Vam3p structure reveals conserved and divergent properties of syntaxins. *Nat Struct Biol* 8, 258-64 (2001)
121. Carr C. M., E. Grote, M. Munson, F. M. Hughson & P. J. Novick: Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J Cell Biol* 146, 333-44 (1999)
122. Ungermann C., K. Sato & W. Wickner: Defining the functions of trans-SNARE pairs. *Nature* 396, 543-8 (1998)
123. Yang B., L. Gonzalez Jr, R. Prekeris, M. Steegmaier, R. J. Advani & R. H. Scheller: SNARE interactions are not selective. Implications for membrane fusion specificity. *J Biol Chem* 274, 5649-53 (1999)
124. Pellegrini L. L., V. O'Connor, F. Lottspeich & H. Betz: Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion. *EMBO J* 14, 4705-13 (1995)
125. Liu Y. & C. Barlowe: Analysis of Sec22p in Endoplasmic Reticulum/Golgi Transport Reveals Cellular Redundancy in SNARE Protein Function. *Mol Biol Cell* 13, 3314-24 (2002)
126. Bhattacharya S., B. A. Stewart, B. A. Niemeyer, R. W. Burgess, B. D. McCabe, P. Lin, G. Boulianne, C. J. O'Kane & T. L. Schwarz: Members of the synaptobrevin/vesicle-associated membrane protein (VAMP) family in Drosophila are functionally interchangeable in vivo for neurotransmitter release and cell viability. *Proc Natl Acad Sci U S A* 99, 13867-72 (2002)
127. von Mollard G. F., S. F. Nothwehr & T. H. Stevens: The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p. *J Cell Biol* 137, 1511-24 (1997)
128. McNew J. A., F. Parlati, R. Fukuda, R. J. Johnston, K. Paz, F. Paumet, T. H. Sollner & J. E. Rothman: Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* 407, 153-9 (2000)
129. Parlati F., J. A. McNew, R. Fukuda, R. Miller, T. H. Sollner & J. E. Rothman: Topological restriction of SNARE-dependent membrane fusion. *Nature* 407, 194-8 (2000)
130. Hsu S. C., A. E. Ting, C. D. Hazuka, S. Davanger, J. W. Kenny, Y. Kee & R. H. Scheller: The mammalian brain rsec6/8 complex. *Neuron* 17, 1209-19 (1996)
131. Sonnichsen B., M. Lowe, T. Levine, E. Jamsa, B. Dirac-Svejstrup & G. Warren: A role for giantin in docking COPI vesicles to Golgi membranes. *J Cell Biol* 140, 1013-21 (1998)
132. Nakamura N., M. Lowe, T. P. Levine, C. Rabouille & G. Warren: The vesicle docking protein p115 binds GM130, a cis-Golgi matrix protein, in a mitotically regulated manner. *Cell* 89, 445-55 (1997)
133. Waters M. G. & S. R. Pfeffer: Membrane tethering in intracellular transport. *Curr Opin Cell Biol* 11, 453-9 (1999)
134. Waters M. G. & F. M. Hughson: Membrane tethering and fusion in the secretory and endocytic pathways. *Traffic* 1, 588-97 (2000)
135. Elferink L. A., W. S. Trimble & R. H. Scheller: Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J Biol Chem* 264, 11061-4 (1989)
136. McMahon H. T., Y. A. Ushkaryov, L. Edelmann, E. Link, T. Binz, H. Niemann, R. Jahn & T. C. Sudhof: Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* 364, 346-9 (1993)
137. Volchuk A., R. Sargeant, S. Sumitani, Z. Liu, L. He & A. Klip: Cellubrevin is a resident protein of insulin-sensitive GLUT4 glucose transporter vesicles in 3T3-L1 adipocytes. *J Biol Chem* 270, 8233-40 (1995)
138. Calakos N., M. K. Bennett, K. E. Peterson & R. H. Scheller: Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* 263, 1146-9 (1994)
139. Tellam J. T., S. L. Macaulay, S. McIntosh, D. R. Hewish, C. W. Ward & D. E. James: Characterization of Munc-18c and syntaxin-4 in 3T3-L1 adipocytes. Putative role in insulin-dependent movement of GLUT-4. *J Biol Chem* 272, 6179-86 (1997)
140. Timmers K. I., A. E. Clark, M. Omatsu-Kanbe, S. W. Whiteheart, M. K. Bennett, G. D. Holman & S. W. Cushman: Identification of SNAP receptors in rat adipose cell membrane fractions and in SNARE complexes co-immunoprecipitated with epitope-tagged N- ethylmaleimide-sensitive fusion protein. *Biochem J* 320 (Pt 2), 429-36 (1996)
141. St-Denis J. F., J. P. Cabaniols, S. W. Cushman & P. A. Roche: SNAP-23 participates in SNARE complex assembly in rat adipose cells. *Biochem J* 338 (Pt 3), 709-15 (1999)
142. Olson A. L., J. B. Knight & J. E. Pessin: Syntaxin 4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes. *Mol Cell Biol* 17, 2425-35 (1997)
143. Cheatham B., A. Volchuk, C. R. Kahn, L. Wang, C. J. Rhodes & A. Klip: Insulin-stimulated translocation of GLUT4 glucose transporters requires SNARE-complex proteins. *Proc Natl Acad Sci U S A* 93, 15169-73 (1996)
144. Macaulay S. L., D. R. Hewish, K. H. Gough, V. Stoichevska, S. F. MacPherson, M. Jagadish & C. W.

Ward: Functional studies in 3T3L1 cells support a role for SNARE proteins in insulin stimulation of GLUT4 translocation. *Biochem J* 324 (Pt 1), 217-24 (1997)
145. Aledo J. C., E. Hajduch, F. Darakhshan & H. S. Hundal: Analyses of the co-localization of cellubrevin and the GLUT4 glucose transporter in rat and human insulin-responsive tissues. *FEBS Lett* 395, 211-6 (1996)
146. Sevilla L., E. Tomas, P. Munoz, A. Guma, Y. Fischer, J. Thomas, B. Ruiz-Montasell, X. Testar, M. Palacin, J. Blasi & A. Zorzano: Characterization of two distinct intracellular GLUT4 membrane populations in muscle fiber. Differential protein composition and sensitivity to insulin. *Endocrinology* 138, 3006-15 (1997)
147. Randhawa V. K., P. J. Bilan, Z. A. Khayat, N. Daneman, Z. Liu, T. Ramlal, A. Volchuk, X. R. Peng, T. Coppola, R. Regazzi, W. S. Trimble & A. Klip: VAMP2, but not VAMP3/cellubrevin, mediates insulin-dependent incorporation of GLUT4 into the plasma membrane of L6 myoblasts. *Mol Biol Cell* 11, 2403-17 (2000)
148. Yang C., S. Mora, J. W. Ryder, K. J. Coker, P. Hansen, L. A. Allen & J. E. Pessin: VAMP3 null mice display normal constitutive, insulin- and exercise- regulated vesicle trafficking. *Mol Cell Biol* 21, 1573-80 (2001)
149. Jo I., H. W. Harris, A. M. Amendt-Raduege, R. R. Majewski & T. G. Hammond: Rat kidney papilla contains abundant synaptobrevin protein that participates in the fusion of antidiuretic hormone-regulated water channel-containing endosomes in vitro. *Proc Natl Acad Sci U S A* 92, 1876-80 (1995)
150. Karvar S., X. Yao, J. G. Duman, K. Hybiske, Y. Liu & J. G. Forte: Intracellular distribution and functional importance of vesicle- associated membrane protein 2 in gastric parietal cells. *Gastroenterology* 123, 281-90 (2002)
151. Regazzi R., C. B. Wollheim, J. Lang, J. M. Theler, O. Rossetto, C. Montecucco, K. Sadoul, U. Weller, M. Palmer & B. Thorens: VAMP-2 and cellubrevin are expressed in pancreatic beta-cells and are essential for Ca(2+)-but not for GTP gamma S-induced insulin secretion. *EMBO J* 14, 2723-30 (1995)
152. Martincic I., M. E. Peralta & J. K. Ngsee: Isolation and characterization of a dual prenylated Rab and VAMP2 receptor. *J Biol Chem* 272, 26991-8 (1997)
153. Hutt D. M., L. F. Da-Silva, L. H. Chang, D. C. Prosser & J. K. Ngsee: PRA1 inhibits the extraction of membrane-bound rab GTPase by GDI1. *J Biol Chem* 275, 18511-9 (2000)
154. Skehel P. A., B. A. Armitage, D. Bartsch, Y. Hu, B. K. Kaang, S. A. Siegelbaum, E. R. Kandel & K. C. Martin: Proteins functioning in synaptic transmission at the sensory to motor synapse of Aplysia. *Neuropharmacology* 34, 1379-85 (1995)
155. Weir M. L., A. Klip & W. S. Trimble: Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. *Biochem J* 333 (Pt 2), 247-51 (1998)
156. Pennetta G., P. Hiesinger, R. Fabian-Fine, I. Meinertzhagen & H. Bellen: Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* 35, 291-306 (2002)
157. Foster L. J., M. L. Weir, D. Y. Lim, Z. Liu, W. S. Trimble & A. Klip: A functional role for VAP-33 in insulin-stimulated GLUT4 traffic. *Traffic* 1, 512-21 (2000)
158. Haass N. K., M. A. Kartenbeck & R. E. Leube: Pantophysin is a ubiquitously expressed synaptophysin homologue and defines constitutive transport vesicles. *J Cell Biol* 134, 731-46 (1996)
159. Brooks C. C., P. E. Scherer, K. Cleveland, J. L. Whittemore, H. F. Lodish & B. Cheatham: Pantophysin is a phosphoprotein component of adipocyte transport vesicles and associates with GLUT4-containing vesicles. *J Biol Chem* 275, 2029-36 (2000)
160. Calakos N. & R. H. Scheller: Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. *J Biol Chem* 269, 24534-7 (1994)
161. Sumitani S., T. Ramlal, Z. Liu & A. Klip: Expression of syntaxin 4 in rat skeletal muscle and rat skeletal muscle cells in culture. *Biochem Biophys Res Commun* 213, 462-8 (1995)
162. Jagadish M. N., C. S. Fernandez, D. R. Hewish, S. L. Macaulay, K. H. Gough, J. Grusovin, A. Verkuylen, L. Cosgrove, A. Alafaci, M. J. Frenkel & C. W. Ward: Insulin-responsive tissues contain the core complex protein SNAP-25 (synaptosomal-associated protein 25) A and B isoforms in addition to syntaxin 4 and synaptobrevins 1 and 2. *Biochem J* 317 (Pt 3), 945-54 (1996)
163. Torrejon-Escribano B., I. Gomez de Aranda & J. Blasi: SNARE expression and distribution during 3T3-L1 adipocyte differentiation. *FEBS Lett* 512, 275-81 (2002)
164. Volchuk A., Q. Wang, H. S. Ewart, Z. Liu, L. He, M. K. Bennett & A. Klip: Syntaxin 4 in 3T3-L1 adipocytes: regulation by insulin and participation in insulin-dependent glucose transport. *Mol Biol Cell* 7, 1075-82 (1996)
165. Munson M., X. Chen, A. E. Cocina, S. M. Schultz & F. M. Hughson: Interactions within the yeast t-SNARE Sso1p that control SNARE complex assembly. *Nat Struct Biol* 7, 894-902 (2000)
166. Misura K. M., R. H. Scheller & W. I. Weis: Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* 404, 355-62 (2000)
167. Thurmond D. C., B. P. Ceresa, S. Okada, J. S. Elmendorf, K. Coker & J. E. Pessin: Regulation of insulin-stimulated GLUT4 translocation by Munc18c in 3T3L1 adipocytes. *J Biol Chem* 273, 33876-83 (1998)
168. Araki S., Y. Tamori, M. Kawanishi, H. Shinoda, J. Masugi, H. Mori, T. Niki, H. Okazawa, T. Kubota & M. Kasuga : Inhibition of the binding of SNAP-23 to syntaxin 4 by Munc18c. *Biochem Biophys Res Commun* 234, 257-62 (1997)
169. Min J., S. Okada, M. Kanzaki, J. S. Elmendorf, K. J. Coker, B. P. Ceresa, L. J. Syu, Y. Noda, A. R. Saltiel & J. E. Pessin: Synip: a novel insulin-regulated syntaxin 4-binding protein mediating GLUT4 translocation in adipocytes. *Mol Cell* 3, 751-60 (1999)
170. Chamberlain L. H., M. E. Graham, S. Kane, J. L. Jackson, V. H. Maier, R. D. Burgoyne & G. W. Gould: The synaptic vesicle protein, cysteine-string protein, is associated with the plasma membrane in 3T3-L1 adipocytes and interacts with syntaxin 4. *J Cell Sci* 114, 445-55 (2001)
171. Wang G., J. W. Witkin, G. Hao, V. A. Bankaitis, P. E. Scherer & G. Baldini: Syndet is a novel SNAP-25 related protein expressed in many tissues. *J Cell Sci* 110 (Pt 4), 505-13 (1997)
172. Gonzalo S., W. K. Greentree & M. E. Linder: SNAP-25 is targeted to the plasma membrane through a novel

membrane- binding domain. *J Biol Chem* 274, 21313-8 (1999)
173. Vogel K. & P. A. Roche: SNAP-23 and SNAP-25 are palmitoylated in vivo . *Biochem Biophys Res Commun* 258, 407-10 (1999)
174. Koticha D. K., S. J. Huddleston, J. W. Witkin & G. Baldini: Role of the cysteine-rich domain of the t-SNARE component, SYNDET, in membrane binding and subcellular localization. *J Biol Chem* 274, 9053-60 (1999)
175. Foster L. J., B. Yeung, M. Mohtashami, K. Ross, W. S. Trimble & A. Klip: Binary interactions of the SNARE proteins syntaxin-4, SNAP23, and VAMP- 2 and their regulation by phosphorylation. *Biochemistry* 37, 11089-96 (1998)
176. Rea S., L. B. Martin, S. McIntosh, S. L. Macaulay, T. Ramsdale, G. Baldini & D. E. James: Syndet, an adipocyte target SNARE involved in the insulin-induced translocation of GLUT4 to the cell surface. *J Biol Chem* 273, 18784-92 (1998)
177. Hickson G. R., L. H. Chamberlain, V. H. Maier & G. W. Gould: Quantification of SNARE protein levels in 3T3-L1 adipocytes: implications for insulin-stimulated glucose transport. *Biochem Biophys Res Commun* 270, 841-5 (2000)
178. Foster L. J., K. Yaworsky, W. S. Trimble & A. Klip: SNAP23 promotes insulin-dependent glucose uptake in 3T3-L1 adipocytes: possible interaction with cytoskeleton. *Am J Physiol* 276, C1108-14 (1999)
179. Kawanishi M., Y. Tamori, H. Okazawa, S. Araki, H. Shinoda & M. Kasuga: Role of SNAP23 in insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. *J Biol Chem* 275, 8240-7 (2000)
180. Cabaniols J. P., V. Ravichandran & P. A. Roche: Phosphorylation of SNAP-23 by the novel kinase SNAK regulates t-SNARE complex assembly. *Mol Biol Cell* 10, 4033-41 (1999)
181. Bock J. B., H. T. Matern, A. A. Peden & R. H. Scheller: A genomic perspective on membrane compartment organization. *Nature* 409, 839-41 (2001)
182. Hata Y., C. A. Slaughter & T. C. Sudhof: Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature* 366, 347-51 (1993)
183. Pevsner J., S. C. Hsu & R. H. Scheller: n-Sec1: a neural-specific syntaxin-binding protein. *Proc Natl Acad Sci U S A* 91, 1445-9 (1994)
184. Garcia E. P., E. Gatti, M. Butler, J. Burton & P. De Camilli: A rat brain Sec1 homologue related to Rop and UNC18 interacts with syntaxin. *Proc Natl Acad Sci U S A* 91, 2003-7 (1994)
185. Hata Y. & T. C. Sudhof: A novel ubiquitous form of Munc-18 interacts with multiple syntaxins. Use of the yeast two-hybrid system to study interactions between proteins involved in membrane traffic. *J Biol Chem* 270, 13022-8 (1995)
186. Tellam J. T., S. McIntosh & D. E. James: Molecular identification of two novel Munc-18 isoforms expressed in non- neuronal tissues. *J Biol Chem* 270, 5857-63 (1995)
187. Khan A. H., D. C. Thurmond, C. Yang, B. P. Ceresa, C. D. Sigmund & J. E. Pessin: Munc18c regulates insulin-stimulated glut4 translocation to the transverse tubules in skeletal muscle. *J Biol Chem* 276, 4063-9 (2001)
188. Novick P. & R. Schekman: Secretion and cell-surface growth are blocked in a temperature- sensitive mutant of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 76, 1858-62 (1979)
189. Hosono R., S. Hekimi, Y. Kamiya, T. Sassa, S. Murakami, K. Nishiwaki, J. Miwa, A. Taketo & K. I. Kodaira: The unc-18 gene encodes a novel protein affecting the kinetics of acetylcholine metabolism in the nematode Caenorhabditis elegans. *J Neurochem* 58, 1517-25 (1992)
190. Harrison S. D., K. Broadie, J. van de Goor & G. M. Rubin: Mutations in the Drosophila Rop gene suggest a function in general secretion and synaptic transmission. *Neuron* 13, 555-66 (1994)
191. Verhage M., A. S. Maia, J. J. Plomp, A. B. Brussaard, J. H. Heeroma, H. Vermeer, R. F. Toonen, R. E. Hammer, T. K. van den Berg, M. Missler, H. J. Geuze & T. C. Sudhof: Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287, 864-9 (2000)
192. Rowe J., N. Corradi, M. L. Malosio, E. Taverna, P. Halban, J. Meldolesi & P. Rosa: Blockade of membrane transport and disassembly of the Golgi complex by expression of syntaxin 1A in neurosecretion-incompetent cells: prevention by rbSEC1. *J Cell Sci* 112 (Pt 12), 1865-77 (1999)
193. Rowe J., F. Calegari, E. Taverna, R. Longhi & P. Rosa: Syntaxin 1A is delivered to the apical and basolateral domains of epithelial cells: the role of munc-18 proteins. *J Cell Sci* 114, 3323-32 (2001)
194. Perez-Branguli F., A. Muhaisen & J. Blasi: Munc 18a binding to syntaxin 1A and 1B isoforms defines its localization at the plasma membrane and blocks SNARE assembly in a three-hybrid system assay. *Mol Cell Neurosci* 20, 169-80 (2002)
195. Bryant N. J. & D. E. James: Vps45p stabilizes the syntaxin homologue Tlg2p and positively regulates SNARE complex formation. *EMBO J* 20, 3380-8 (2001)
196. Tamori Y., M. Kawanishi, T. Niki, H. Shinoda, S. Araki, H. Okazawa & M. Kasuga: Inhibition of insulin-induced GLUT4 translocation by Munc18c through interaction with syntaxin4 in 3T3-L1 adipocytes. *J Biol Chem* 273, 19740-6 (1998)
197. Schulze K. L., J. T. Littleton, A. Salzberg, N. Halachmi, M. Stern, Z. Lev & H. J. Bellen: rop, a Drosophila homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron* 13, 1099-108 (1994)
198. Wu M. N., T. Fergestad, T. E. Lloyd, Y. He, K. Broadie & H. J. Bellen: Syntaxin 1A interacts with multiple exocytic proteins to regulate neurotransmitter release in vivo. *Neuron* 23, 593-605 (1999)
199. Macaulay S., J. Grusovin, V. Stoichevska, J. Ryan, L. Castelli & C. Ward: Cellular munc18c levels can modulate glucose transport rate and GLUT4 translocation in 3T3L1 cells. *FEBS Lett* 528, 154 (2002)
200. Zhang W., A. Efanov, S. N. Yang, G. Fried, S. Kolare, H. Brown, S. Zaitsev, P. O. Berggren & B. Meister: Munc-18 associates with syntaxin and serves as a negative regulator of exocytosis in the pancreatic beta -cell. *J Biol Chem* 275, 41521-7 (2000)
201. Yang B., M. Steegmaier, L. C. Gonzalez Jr & R. H. Scheller: nSec1 binds a closed conformation of syntaxin1A. *J Cell Biol* 148, 247-52 (2000)

202. Fujita Y., T. Sasaki, K. Fukui, H. Kotani, T. Kimura, Y. Hata, T. C. Sudhof, R. H. Scheller & Y. Takai: Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J Biol Chem* 271, 7265-8 (1996)
203. Fletcher A. I., R. Shuang, D. R. Giovannucci, L. Zhang, M. A. Bittner & E. L. Stuenkel: Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. *J Biol Chem* 274, 4027-35 (1999)
204. Thurmond D. C., M. Kanzaki, A. H. Khan & J. E. Pessin: Munc18c function is required for insulin-stimulated plasma membrane fusion of GLUT4 and insulin-responsive amino peptidase storage vesicles. *Mol Cell Biol* 20, 379-88 (2000)
205. Brummer M. H., K. J. Kivinen, J. Jantti, J. Toikkanen, H. Soderlund & S. Keranen: Characterization of the sec1-1 and sec1-11 mutations. *Yeast* 18, 1525-36 (2001)
206. Dulubova I., T. Yamaguchi, Y. Gao, S. W. Min, I. Huryeva, T. C. Sudhof & J. Rizo: How Tlg2p/syntaxin 16 'snares' Vps45. *EMBO J* 21, 3620-31 (2002)
207. Yamaguchi T., I. Dulubova, S. W. Min, X. Chen, J. Rizo & T. C. Sudhof: Sly1 binds to Golgi and ER syntaxins via a conserved N-terminal peptide motif. *Dev Cell* 2, 295-305 (2002)
208. Peng R. & D. Gallwitz: Sly1 protein bound to Golgi syntaxin Sed5p allows assembly and contributes to specificity of SNARE fusion complexes. *J Cell Biol* 157, 645-55 (2002)
209. Bracher A. & W. Weissenhorn: Structural basis for the Golgi membrane recruitment of Sly1p by Sed5p. *EMBO J* 21, 6114-24 (2002)
210. Zerial M. & H. McBride: Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2, 107-17 (2001)
211. McLauchlan H., J. Newell, N. Morrice, A. Osborne, M. West & E. Smythe: A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr Biol* 8, 34-45 (1998)
212. Carroll K. S., J. Hanna, I. Simon, J. Krise, P. Barbero & S. R. Pfeffer: Role of Rab9 GTPase in facilitating receptor recruitment by TIP47. *Science* 292, 1373-6 (2001)
213. Short B., C. Preisinger, J. Schaletzky, R. Kopajtich & F. A. Barr: The Rab6 GTPase Regulates Recruitment of the Dynactin Complex to Golgi Membranes. *Curr Biol* 12, 1792-5 (2002)
214. Strom M., A. N. Hume, A. K. Tarafder, E. Barkagianni & M. C. Seabra: A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. *J Biol Chem* 277, 25423-30 (2002)
215. Gmachl M. J. & C. Wimmer: Sequential involvement of p115, SNAREs, and Rab proteins in intra-Golgi protein transport. *J Biol Chem* 276, 18178-84 (2001)
216. De Antoni A., J. Schmitzova, H. H. Trepte, D. Gallwitz & S. Albert: Significance of GTP Hydrolysis in Ypt1p-regulated Endoplasmic Reticulum to Golgi Transport Revealed by the Analysis of Two Novel Ypt1-GAPs. *J Biol Chem* 277, 41023-31 (2002)
217. Brickner J. H., J. M. Blanchette, G. Sipos & R. S. Fuller: The Tlg SNARE complex is required for TGN homotypic fusion. *J Cell Biol* 155, 969-78 (2001)
218. Lawe D. C., A. Chawla, E. Merithew, J. Dumas, W. Carrington, K. Fogarty, L. Lifshitz, R. Tuft, D. Lambright & S. Corvera: Sequential roles for phosphatidylinositol 3-phosphate and Rab5 in tethering and fusion of early endosomes via their interaction with EEA1. *J Biol Chem* 277, 8611-7 (2002)
219. Simonsen A., J. M. Gaullier, A. D'Arrigo & H. Stenmark: The Rab5 effector EEA1 interacts directly with syntaxin-6. *J Biol Chem* 274, 28857-60 (1999)
220. Li L., W. Omata, I. Kojima & H. Shibata: Direct interaction of Rab4 with syntaxin 4. *J Biol Chem* 276, 5265-73 (2001)
221. Shibata H., W. Omata & I. Kojima: Insulin stimulates guanine nucleotide exchange on Rab4 via a wortmannin-sensitive signaling pathway in rat adipocytes. *J Biol Chem* 272, 14542-6 (1997)
222. Dascher C., R. Ossig, D. Gallwitz & H. D. Schmitt: Identification and structure of four yeast genes (SLY) that are able to suppress the functional loss of YPT1, a member of the RAS superfamily. *Mol Cell Biol* 11, 872-85 (1991)
223. Nielsen E., S. Christoforidis, S. Uttenweiler-Joseph, M. Miaczynska, F. Dewitte, M. Wilm, B. Hoflack & M. Zerial: Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. *J Cell Biol* 151, 601-12 (2000)
224. Wurmser A. E., T. K. Sato & S. D. Emr: New component of the vacuolar class C-Vps complex couples nucleotide exchange on the Ypt7 GTPase to SNARE-dependent docking and fusion. *J Cell Biol* 151, 551-62 (2000)
225. Seals D. F., G. Eitzen, N. Margolis, W. T. Wickner & A. Price: A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc Natl Acad Sci U S A* 97, 9402-7 (2000)
226. Tall G. G., H. Hama, D. B. DeWald & B. F. Horazdovsky: The phosphatidylinositol 3-phosphate binding protein Vac1p interacts with a Rab GTPase and a Sec1p homologue to facilitate vesicle-mediated vacuolar protein sorting. *Mol Biol Cell* 10, 1873-89 (1999)
227. Sato T. K., P. Rehling, M. R. Peterson & S. D. Emr: Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion. *Mol Cell* 6, 661-71 (2000)
228. Trischler M., W. Stoorvogel & O. Ullrich: Biochemical analysis of distinct Rab5- and Rab11-positive endosomes along the transferrin pathway. *J Cell Sci* 112 (Pt 24), 4773-83 (1999)
229. Kessler A., E. Tomas, D. Immler, H. E. Meyer, A. Zorzano & J. Eckel: Rab11 is associated with GLUT4-containing vesicles and redistributes in response to insulin. *Diabetologia* 43, 1518-27 (2000)
230. Zeng J., M. Ren, D. Gravotta, C. De Lemos-Chiarandini, M. Lui, H. Erdjument-Bromage, P. Tempst, G. Xu, T. H. Shen, T. Morimoto, M. Adesnik & D. D. Sabatini: Identification of a putative effector protein for rab11 that participates in transferrin recycling. *Proc Natl Acad Sci U S A* 96, 2840-5 (1999)
231. Mammoto A., T. Ohtsuka, I. Hotta, T. Sasaki & Y. Takai: Rab11BP/Rabphilin-11, a downstream target of rab11 small G protein implicated in vesicle recycling. *J Biol Chem* 274, 25517-24 (1999)
232. van der Sluijs P., M. Hull, P. Webster, P. Male, B. Goud & I. Mellman: The small GTP-binding protein rab4

controls an early sorting event on the endocytic pathway. *Cell* 70, 729-40 (1992)
233. Mohrmann K., L. Gerez, V. Oorschot, J. Klumperman & P. van der Sluijs: Rab4 function in membrane recycling from early endosomes depends on a membrane to cytoplasm cycle. *J Biol Chem* 277, 32029-35 (2002)
234. Cormont M., J. F. Tanti, A. Zahraoui, E. Van Obberghen, A. Tavitian & Y. Le Marchand-Brustel: Insulin and okadaic acid induce Rab4 redistribution in adipocytes. *J Biol Chem* 268, 19491-7 (1993)
235. Aledo J. C., F. Darakhshan & H. S. Hundal: Rab4, but not the transferrin receptor, is colocalized with GLUT4 in an insulin-sensitive intracellular compartment in rat skeletal muscle. *Biochem Biophys Res Commun* 215, 321-8 (1995)
236. Shibata H., W. Omata, Y. Suzuki, S. Tanaka & I. Kojima: A synthetic peptide corresponding to the Rab4 hypervariable carboxyl- terminal domain inhibits insulin action on glucose transport in rat adipocytes. *J Biol Chem* 271, 9704-9 (1996)
237. Cormont M., M. N. Bortoluzzi, N. Gautier, M. Mari, E. van Obberghen & Y. Le Marchand-Brustel: Potential role of Rab4 in the regulation of subcellular localization of Glut4 in adipocytes. *Mol Cell Biol* 16, 6879-86 (1996)
238. Goalstone M. L., J. W. Leitner, I. Golovchenko, M. R. Stjernholm, M. Cormont, Y. Le Marchand-Brustel & B. Draznin: Insulin promotes phosphorylation and activation of geranylgeranyltransferase II. Studies with geranylgeranylation of rab-3 and rab-4. *J Biol Chem* 274, 2880-4 (1999)

Key Words: GLUT4, SNARE, Syntaxin, VAMP, SNAP23, Munc18c, Insulin Action, Glucose Transport, Review

Send correspondence to: Dr S. Lance Macaulay, CSIRO-Health Sciences and Nutrition, 343 Royal Pde, Parkville, Victoria, Australia 3052, Tel: 61-3-96627335, Fax: 61-3-96627101, E-mail: Lance.Macaulay@csiro.au