SNARE phosphoryation: a control mechanism for insulin-stimulated glucose transport and other regulated exocytic events.

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Abbreviations

CDK5	Cyclin-dependant kinase 5
GLUT4	Glucose Transporter Type 4
GSV	GLUT4 Storage Vesicle
LRRK2	Leucine-rich repeat kinase 2
MC	Mast cell
MP	Myosin Phosphatase
MYPT1	Myosin phosphatase targeting subunit
NSF	N-ethylmaleimide sensitive factor
PKA	Protein Kinase A
PKC	Protein Kinase C
PKCB	Protein Kinase C Beta
ROK	RhoA-activated kinase
SDS	Sodium dodecyl sulfate
SM	Sec1/Munc18
SNAP 23	Synaptosomal-associated protein 23
SNAP 25	Synaptosomal-associated protein 25
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
VAMP	Vesicle associated membrane protein

Summary

Trafficking within eukaryotic cells is a complex and highly regulated process; events such as recycling of plasma membrane receptors, formation of multivesicular bodies, regulated release of hormones and delivery of proteins to membranes all require directionality and specificity. The underpinning processes, including cargo selection, membrane fusion, trafficking flow and timing are controlled by a variety of molecular mechanisms and engage multiple families of lipids and proteins. Here we will focus on control of trafficking processes

via the action of the SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) family of proteins, in particular their regulation by phosphorylation. We will describe how these proteins are controlled in a range of regulated trafficking events, with particular emphasis on the insulin-stimulated delivery of glucose transporters to the surface of adipose and muscle cells. Here, we focus on a few examples of SNARE-phosphorylation which exemplify distinct ways in which SNARE machinery phosphorylation may regulate membrane fusion.

1. SNARE proteins – a brief overview.

Membrane trafficking requires a transport vesicle to reach the correct target membrane, dock there, and fuse with the membrane (1,2). The fusion step is commonly considered the final stage in vesicle trafficking, and can be entirely driven by SNARE proteins *in vitro* (3). The SNARE family is large: there are 24 members in yeast and over 60 in humans. SNAREs vary considerably in size, but all are characterised by the presence of a common motif, the SNARE motif, of around 60 amino acids arranged in heptad repeats and are anchored to the membrane by a C-terminal transmembrane domain or in some cases by palmitoylation. SNAREs are generally divided into two types, the vesicle (v-) SNARE and target (t-) SNARE (other nomenclatures have been proposed –(4)). SNAREs drive membrane fusion via the reversible assembly of v- and t-SNAREs into a so-called *trans* SNARE complex (or ternary complex); this is comprised of a helical bundle of four complimentary SNARE motifs which are arranged in parallel, held together by 16 layers of interacting amino acids (see Figure 1) (5–7). The formation of the *trans*-SNARE complex is energetically favourable; indeed many SNARE complexes are heat and SDS resistant and the formation of this complex provides the energy to drive membrane fusion (8). The SNARE hypothesis posits that a vesicle carrying a particular v-SNARE will only fuse with membranes that have specific complimentary t-SNARE. These complementary SNARE motifs associate by the 'zippering' of the SNARE motifs into the trans SNARE complex depicted in figure 1. This overcomes the opposing forces by the two lipid bilayers facilitating membrane fusion. Hence, SNAREs provide the energy for fusion and likely contribute to the specificity of the trafficking event (although the latter clearly engages other regulatory molecules).

The most extensively studied SNARE proteins are found in the brain, which is reliant on the constant release of neurotransmitters in the synaptic space in response to appropriate stimuli – so-called regulated exocytosis (8). There are many other examples of how SNARE-mediated membrane fusion regulates important cellular processes. These include insulin dependent delivery of GLUT4 to the surface of adipocytes and exocytosis of secretory granules in mast cells in response to an allergen (9–11). These tightly regulated exocytic events rely on SNAREs being primed and/or responding to a signal. Recent research has highlighted a role of phosphorylation in control of SNARE protein function. Phosphorylation has been shown to mediate effects at multiple stages of the SNARE protein cycle, including regulation of binding of SNARE-interacting factors, the 'zippering' of the trans SNARE complex and modulating the structure of the SNARE protein (see Figure 1; Table 1). Although SNARE phosphorylation was first identified as a regulatory mechanism in yeast (12,13), this review will consider some recent examples of these regulatory mechanisms, in mammalian cells.

Table 1: Recent examples of SNARE phospho-regulatory mechanisms.

Target	Kinase/ Phosphatase	AA	Effect	Reference
Munc-18	PKC CDK5	S306, S313, Y521, S574		(14) (15,16)
NSF	PKC LRRK2	S237 T645	Increased ATPase activity and improved trans-SNARE complex disassembly	(17) (18)
SNAP-23	?	T102, S95, and S120	102 facilitates PM association. 95/120 facilitates translocation	(10,19,20)
SNAP-25	ROK / MP PKC PKA	T138 T138, S187 T138	ROK inhibits synaptosomal exocytosis, MP acts as a balance to increase release. PKC/PKA enhances regulates exocytosis	(21–24)
Syntaxin-3	?	T14	Negative effect on Munc18-2 binding and reduces exocytosis in MC	(25)
Syntaxin-4	Insulin Receptor dependant	Y115, Y251	Promotes SNARE complex assembly	(26)

Syntaxin-16	Insulin Receptor dependant	T7	Controls entry of GLUT4 into GSVs	Our unpublished work.
VAMP 8	PKCB dependent	T47, T53, S54, and S61	Inhibits trans-SNARE zippering	(27,28)

2. Phospho-control of SNARE protein function.

2.1 SNARE motif phosphorylation

Direct phosphorylation of residues within the SNARE motif can inhibit SNARE mediated vesicle fusion and SNARE complex formation (12,13). Phosphorylation of sites near the C-terminal end of the SNARE motif are hypothesised to impair the zippering interaction (29) and consequently the helical bundle cannot fully drive membrane fusion (see Figure 1). A recent example provides a compelling illustration of this kind of regulatory mechanism. Phosphorylation of the v-SNARE VAMP8 within the SNARE motif in a protein kinase C-β dependent manner inhibits secretion from Mast cells. The phosphorylation sites identified (T47, T53, S54, and S61) lie within the centre of the helical bundle at the C-terminus of the SNARE motif (27,28) (the interested reader is referred to the structural model at PDB:4WY4). Analysis of cells expressing phosphomimetic mutants of VAMP8 revealed that vesicle fusion is impaired in liposome assays and SDS-resistant SNARE complex formation is impaired (27). Total internal reflection fluorescence microscopy revealed that expression of phosphomimetic VAMP8 mutants led to a significant accumulation of vesicles docked but not fused at the plasma membrane after stimulation. Taken together this suggests that the phosphorylation of the VAMP8 SNARE motif does not inhibit the docking step of membrane trafficking, which might involve partial SNARE assembly, but rather may reflect an inhibition of the SNARE complex zippering required for fusion (27). This mode of regulation, where the v-SNARE can act as an inhibitor but is still able to bind with complementary t-SNAREs may allow for decreased fusion kinetics appropriate for this trafficking. Such regulation could also allow for priming of a vesicle at the target membrane, with dephosphorylation acting as a positive regulator. Strikingly, the phospho-regulatory sites are absent in all neuronal v-SNARE isoforms, suggesting that manipulation of secretion via non-neuronal SNARE

phosphorylation cycles might have therapeutic potential (28) and may be a wide-spread regulatory mechanism in non-neuronal tissues. The potential that these phospho-control mechanisms represent novel therapeutic targets in disease such as diabetes or in immunological dysfunction is an exciting proposition.

2.2 Phosphoregulation of a SNARE regulatory protein – N-ethylmaleimide sensitive factor.

The ATPase N-ethylmaleimide sensitive factor (NSF) is a known regulator of SNARE proteins. NSF is thought to act by disassembling the *cis*-SNARE complex after fusion to allow for recycling of the SNARE proteins (see Figure 1) (30,31). Recent work has identified leucine-rich repeat kinase 2 (LRRK2) acting on NSF to control membrane trafficking (17,18,32). *Lrrk2* is a gene known to be associated with Parkinson's disease, and LRRK2 is associated with synaptic membranes, and over-expression or knockdown studies have shown that LRRK2 can significantly impact synaptic vesicle endo/exocytosis (18). A recent study has revealed that LRRK2 directly phosphorylates NSF in the ATP binding pocket of the protein. Phosphorylation of NSF by LRRK2, and also by PKC, results in an increase in ATPase activity (17,18). The increased activity of NSF leads to improved *cis*-SNARE complex disassembly and efficient secretory vesicle fusion (18). Therefore, activity of LRRK2 mediates storage and mobilisation of secretory vesicles in the neuronal synapse through improving NSF ability to recycle SNAREs involved. Such studies hint at complex regulatory networks that can switch the balance between rapidly released exocytic populations and more slowly released pools by regulating the recycling of the SNARE complexes. Whether similar mechanisms operate in other rapidly mobilised vesicle pools, such as insulin granules in the pancreatic beta cell, or are utilised in cellular systems where expcotosis is 'slower' remains to be determined.

2.3 Syntaxin/SM protein interaction.

The Syntaxin family of proteins (also known as Q_a-SNAREs) contain a SNARE motif and a three helical regulatory domain known as the H_{abc} domain (33). For many Syntaxins, including the mammalian neuronal Syntaxin-1a, this H_{abc} domain binds intramolecularly to the SNARE motif region, and this closed conformation is inhibitory to SNARE complex formation (shown in Figure 2) (34). Q_a-SNAREs are regulated in part by their interaction with

the Sec1/Munc18 family (SM) of proteins. SM proteins have multiple roles in exocytosis, including regulating Syntaxin stability, SNARE complexes assembly and also appear to participate in the fusion process by binding the *trans*-SNARE complex (see Figure 2) (35).

Structural studies of the Munc18a/Syntaxin1a complex revealed that the SM protein is an arch shaped molecule that holds the Syntaxin molecule in its closed conformation, known as mode 1 binding (34). However, other members of the SM protein family were found to use an alternative mechanism to bind their cognate syntaxins. In so-called mode 2 binding (see Figure 2), the open conformation of Syntaxin inserts a free N-terminus into a hydrophobic pocket on the SM protein (34). Interruption to mode 2 binding in mammalian cells results in trafficking defects, however complete abolition of this binding in SM proteins Sly1p and Vps45p does not confer additional effects (34). The nature of the SM/SNARE protein interaction is further complicated by a further mode of interaction whereby SM proteins can directly bind to the intact SNARE complex and promote fusion (see Figure 2) (36). These data support the idea that regulating the Syntaxin/SM interaction might be a commonly utilised theme in biology (35,37).

In Mast cells, syntaxin-3 is constitutively phosphorylated at Thr14. This phosphorylation regulates the interaction of this Q_a-SNARE with its cognate SM protein, Munc18-2; phosphorylation of syntaxin-3 results in a change in secondary protein structure, decreased binding to Munc18-2 and results in suppressed exocytic activity (25). Interestingly, there are potential phosphorylation sites within the N-termini of many members of the Q_a family. We have identified Thr-7 as a site of phosphorylation of Synaxtin16; mutation of this site to alanine did not modulate interaction with the cognate SM protein, mVps45. However, mutation to aspartate (phosphomimetic) abrogated the interaction (Berends, Bryant and Gould, unpublished). Such results indicate that phosphorylation of the SM protein interacting peptide at the N-terminus of Q_a-SNAREs might be a commonly used regulatory mechanism.

2.4 Tyrosine Phosphorylation of Sec1/Munc18 proteins.

Insulin plays a central role in regulating whole body glucose homeostasis. Insulin activates its receptor tyrosine kinase and stimulates uptake of glucose from the circulation into adipose tissue and skeletal muscle by facilitating delivery of glucose transporters (GLUT4) to

the plasma membrane. In the absence of insulin, GLUT4 is stored in specialised storage vesicles, known as GLUT4 storage vesicles (GSV's). Following an insulin stimulus, GSV's are trafficked to the cell surface and result in a 10 to 20-fold increase in GLUT4 at the plasma membrane (38). The t-SNAREs involved in GLUT4 trafficking are Syntaxin-4/SNAP-23, and the cognate v-SNARE is VAMP2 (39–41).

It is of interest that both the SM protein Munc18c and its cognate Q_a-SNARE Syntaxin4 are phosphorylated on tyrosine residues in response to insulin. In the case of Munc18c this has been shown to regulate SNARE complex assembly at least *in vitro* (42,43). In response to an insulin stimulus, Munc18c exhibits >10 fold increase in tyrosine phosphorylation in 3T3-L1 adipocytes, specifically at residue 521 (44). Homozygotic disruption of the Munc18c gene in mice 3T3-L1 adipocytes results in enhanced translocation of GLUT4 in response to an insulin stimulus (45). Interestingly, expression of phospho-resistant mutants of Munc18c fail to rescue defective insulin-stimulated glucose transport in 3T3-L1 adipocytes, suggesting that a regulatory mechanism involving tyrosine phosphorylation of the SM protein likely operates *in vivo* (46). As reported by us previously (42), we hypothesise that insulin-stimulated phosphorylation of Munc18c results in increased SNARE complex formation, and may act on a sub-population of Syntaxin4-containing SNARE complexes 'primed' for rapid fusion with the plasma membrane in response to an insulin trigger. In this model, Munc18c acts as a scaffold for SNARE assembly, regulated by direct phosphorylation of Munc18c. The fact that Munc18c is directly phosphorylated by the insulin receptor, at least *in vitro* (38), further hints that sub-cellular localisation of the signalling and trafficking machinery may be co-ordinately regulated.

2.5 Phospho-regulation of SNAP25 family members.

Mast cells are part of the immune response and require the ability to quickly secrete in response to an external signal (10,47,48). The phospho-regulation of SNAREs in this system has recently been investigated. SNAP-23 is a peripheral plasma membrane-associated protein that, together with Syntaxin-4, provides the components of the t-SNARE for Mast cell degranulation. SNAP23 is basally phosphorylated at Thr102 and is inducibly phosphorylated at Ser95 and Ser120 (20). Activation of Mast cells with IgE results in SNAP-23 serine phosphorylation and relocation from the plasma membrane to intracellular

lysosomal membranes. Evidence suggests that Thr102 phosphorylation is important for membrane association of SNAP-23 and Ser95/120 phosphorylation is required for translocation to lysosomal membranes in response to Mast Cell activation (19). These results show that not only can phosphorylation be responsible for regulating trans-SNARE formation but can also be critical for localisation within the cell and thus indirectly regulate exocytosis.

Kinase regulation of SNARE activity has long been the focus of researchers, however there has been recent interest in phosphatase activity. SNAP-25 has two identified phospho-sites T138 and S187 (23,24). Myosin phosphatase has been shown to co-immunoprecipitate with SNAP-25 via the myosin phosphatase targeting subunit (MYPT1). MYPT1 dephosphorylates T138, an action shown to increase exocytosis of synaptosomes (21). This is in contrast to previous evidence from PKC and PKA studies that suggested phosphorylation of this site *increased* SNAP-25 SNARE activity. T138 lies within the SNAP-25/Syntaxin1 interacting region, supporting the idea that phosphorylation has been shown inhibit interaction (21). The role of the phosphatase may be to rapidly switch the balance between positive and negative signals in different regions of the cell. Further work will be required to define the molecular consequences of these processes, but they are included here to emphasise the importance of both phosphorylation and dephosphorylation processes.

3. Summary and perspectives.

The regulation of SNARE complex formation/disassembly offers the potential for rapid regulation of membrane trafficking processes. Phosphorylation control of SNARE complexes is a fast-moving area, and the examples used here only scratch the surface of an interesting field. The studies we highlight here serve to exemplify a number of potential mechanisms that are known to operate at different stages of the SNARE cycle; these studies begin to make links between the activation of signalling pathways and the control of subcellular traffic, and the elucidation of the molecular detail and their dynamics will likely reveal new therapeutic potential in the combatting of a range of human disease.

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Figure Legends.

Figure 1: SNARE driven membrane fusion. The lipid bilayer is an optimal thermodynamic state and therefore membrane fusion requires accessory proteins to facilitate the fusion of two membranes. SNARE proteins are transmembrane proteins (or are palmitoylated). **(A)** As a vesicle arrives at the target membrane the complimentary SNARE motifs facilitate vesicle docking. **(B)** SNAREs are believed to play a role in the docking step which may involve partially assembled SNARE motifs (see text). The complimentary SNARE motifs from the vesicle and target membrane form a *trans*-SNARE complex by parallel arrangement of their SNARE motifs. This complex 'zippers' to form a stable SNARE complex (C) where the tight binding of this complex provides the energy for fusion (D). Phosphorylation of VAMP8 in the SNARE motif has been shown to inhibit the zippering process (see text).) (E) N-ethylmaleimide sensitive factor (NSF) ATPase activity dissembles the *cis*-SNARE complex after fusion, recycling the SNARE proteins. Phosphorylation of NSF has been shown to increase ATPase activity and improve vesicle exocytosis (see text). For brevity, only the SNARE domains of the Q_b and Q_c SNAREs are presented.

Figure 2: Modes of Sec1/Munc18/SNARE interactions. In this figure, different modes of interaction between Syntaxin and Sec1/Munc18 proteins are presented schematically. Mode-1 binding involves the arch-shaped Munc18c binding the 'closed' conformation of Syntaxin4. Mode-2 involves an interaction between the amino-terminus of Syntaxin and a distinct binding pocket on Munc18. A further interaction (Mode-3) between Munc18 and the assembled SNARE complex is thought to potential fusion; the structural basis of this interaction is less well-established. For details, see text. Phosphorylation of either the Syntaxin or the Munc could clearly modulate any or all of these interactions.

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Conflict of Interest:

The authors declare no conflict of interest.

Figure 1

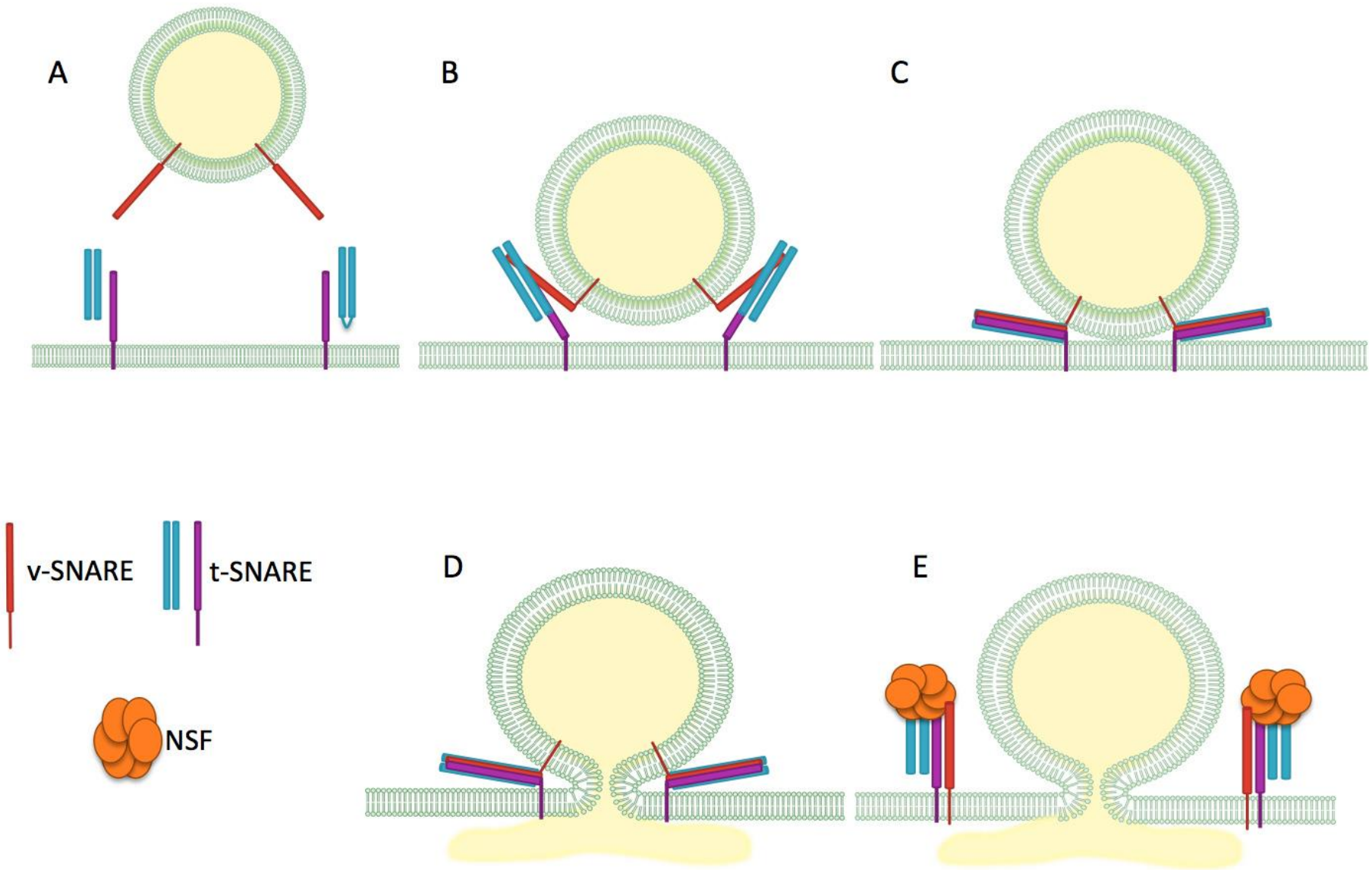

Figure 2