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Glucose transporter 4: cycling, compartments and controversies

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Insulin promotes glucose uptake into muscle and adipose tissues through glucose transporter 4 (GLUT4). In unstimulated cells, rapid endocytosis, slow exocytosis and dynamic or static retention cause GLUT4 to concentrate in early recycling endosomes, the *trans*-Golgi network and vesicle-associated protein 2-containing vesicles. The coordinated action of phosphatidylinositol 3-kinase effectors, protein kinase Akt, atypical protein kinase C (aPKC) and Akt substrate of 160-kDa (AS160), regulates the GLUT4 cycle by affecting its translocation, fusion with the plasma membrane, internalization and sorting. We review the evidence that supports such cycling, evaluate current models proposing static or dynamic retention, and highlight how distinct steps of GLUT4 transport are regulated by insulin signals. In particular, fusion seems to be regulated by aPKC (via munc18) and Akt (via syntaxin4-interacting protein (synip)). AS160 participates in GLUT4 intracellular retention, and possibly fusion, through candidate ras-related GTP-binding protein (Rab)2, Rab8, Rab10 and/or Rab14. The localization of the insulin-sensitive GLUT4 compartment and the precise target of insulin-derived signals remain open for future investigation.
Keywords: glucose uptake; GLUT4 compartments; GLUT4 traffic; insulin signalling; type 2 diabetes mellitus
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Introduction

The global prevalence of type 2 diabetes will reach 300 million cases by 2025 (Zimmet *et al*, 2001). This metabolic disease results from defective tissue sensitivity to insulin and subsequent impairment in insulin secretion. Insulin maintains glucose homeostasis largely by enhancing glucose uptake into muscle and adipose tissues, which is a process mediated by glucose transporter 4 (GLUT4). Since the cloning of GLUT4 in 1989, numerous studies have attempted to dissect the molecular basis of its regulation by insulin and stimuli such as muscle contraction. Most of our understanding of these phenomena stems from studies in cultured cells, as reviewed here. In unstimulated adipose and muscle cells, GLUT4 constitutively cycles to and from the plasma membrane (PM), but the extent of such cycling is currently debated. In both cell types, the steady-state distribution of GLUT4 favours intracellular compartments, and there is a general agreement that insulin largely promotes the exocytic arm of GLUT4 cycling and, to a lesser extent, reduces the endocytic arm (Bryant *et al*, 2002; Rudich & Klip, 2003; Watson *et al*, 2004a). Unlike secretory granule exocytosis, which is unidirectional, regulated GLUT4 cycling is more akin to the regulated secretory exocytosis shown by synaptic vesicles, which, following neurotransmitter release, reform by membrane internalization and protein sorting (Südhof, 2004). Insulin resistance—particularly in skeletal muscle—is associated with insufficient recruitment of GLUT4 to the PM despite normal GLUT4 expression (Björnholm & Zierath, 2005). This finding emphasizes the importance of understanding GLUT4 dynamics in designing strategies to bypass or resolve insulin resistance. The regulation of glucose influx might also involve steps at the level of GLUT4 and hexokinase activities (Antonescu *et al*, 2005; Fueger *et al*, 2005), which are beyond the scope of this review. Here, we focus on the current understanding and ongoing debate regarding GLUT4 cycling.

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Intracellular localization of GLUT4

GLUT4 constitutively cycles to and from the PM through slow exocytosis and fast endocytosis (Satoh *et al*, 1993; Li *et al*, 2001). Diverse microscopy approaches have localized GLUT4 to tubulo-vesicular structures in the perinuclear region and in distinct foci throughout the cytosol (Slot *et al*, 1991; Malide *et al*, 2000). Perinuclear GLUT4 partially colocalizes with markers of the endosomal recycling compartment (ERC), the Golgi complex and the *trans*-Golgi network (TGN; Bryant *et al*, 2002; Ploug & Ralston, 2002). Given the dynamic behaviour of GLUT4, it is not possible to discern by static microscopy analysis whether perinuclear or cytosolic depots encompass the insulin-sensitive

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GLUT4-donor compartment. These and subsequent studies involving the chemical ablation of transferrin receptor (TfR)-containing compartments (Martin *et al*, 1996; Zeigerer *et al*, 2002) using peroxidase-loaded TfR consistently identified two populations of GLUT4 pools: one overlapping with TfR, which is an ERC marker, and one segregating away from it (non-ERC pool). There is consensus that, directly or indirectly, insulin mobilizes GLUT4 from the non-ERC pool, which has therefore been dubbed the GLUT4 'specialized compartment (SC)' or 'GLUT4 storage vesicle' (GSV).

Despite extensive studies, it has been challenging to define the biochemical nature of the SC/GSV. The insulin-responsive aminopeptidase (IRAP) co-segregates with GLUT4 and similarly redistributes to the PM in response to insulin. Although IRAP might regulate the retention/sequestration of GLUT4 vesicles (Hosaka *et al*, 2005), it does not identify a specialized pool of GLUT4 (Keller, 2003), but rather parallels GLUT4 distribution across compartments. A more selective marker of SC/GSV might be vesicle-associated protein 2 (VAMP2), which is a vesicular soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) that is exclusively required for GLUT4 fusion with the membrane in the insulin-stimulated, but not the basal, state. Interestingly, VAMP2 is found only in a subset of GLUT4 vesicles that largely segregate away from TfR (Bryant *et al*, 2002; Watson *et al*, 2004a). Moreover, stimuli such as hypertonic shock and platelet-derived growth factor do not rely on VAMP2 to increase surface GLUT4 levels, and instead depend on the v-SNARE VAMP7 (Randhawa *et al*, 2004; Török *et al*, 2004). Future work should confirm whether the SC/GSV constitutes a pre-formed compartment of VAMP2 and GLUT4 or whether GLUT4 vesicles acquire VAMP2 during insulin-induced translocation to the PM.

How is the SC/GSV formed? The Golgi-localized γ-ear-containing Arf-binding protein GGA is required for nascent GLUT4 sorting from the TGN to the SC/GSV (Watson *et al*, 2004b). GGA interacts with sortilin, which is a TGN and endosomal membrane protein proposed to be necessary and sufficient for the formation of small GLUT4-enriched vesicles and for GLUT4 protein stability (Shi & Kandror, 2005). Accordingly, co-expression of sortilin and GLUT4 tagged with myc7 epitopes in 3T3-L1 fibroblasts generated such vesicles, and, conversely, reducing endogenous sortilin through RNA interference (RNAi) prevented their constitutive formation in 3T3-L1 adipocytes. Whether such vesicles conform to other characteristics of the SC/GSV—such as enrichment in VAMP2—and respond to insulin-derived signals requires further investigation.

GLUT4 cycling: a Ptolemy versus Copernicus analogy

Are there static GLUT4 compartments or do they continuously revolve to and from the PM? Two models have been proposed to explain insulin-responsive GLUT4 storage and cycling in 3T3-L1 adipocytes (Fig 1). Model 1, which is endorsed by McGraw and colleagues, proposes that the entire complement of GLUT4 eventually recycles to the PM in the basal state, that GLUT4 intracellular storage is dynamic and that the SC/GSV is distinct from the furin-positive TGN (Karylowski *et al*, 2004). The last of these conclusions derives from the inability to ablate GLUT4 with peroxidase-loaded furin beyond that caused by peroxidase-loaded TfR. According to this model, insulin promotes two routes for GLUT4 mobilization towards the PM, a direct one from the SC/GSV and an indirect one from the SC/GSV via the ERC. Exit from the ERC is supported by a reduction in insulin-induced GLUT4 translocation to the PM on ablation of TfR-containing compartments, as well as by the participation of the GTPase Rab11 (which controls ERC protein sorting) in the insulin response (Zeigerer *et al*, 2002). The behaviour of GLUT4 in muscle cells is consistent with tenets of this model (Rudich & Klip, 2003). Indeed, in unstimulated muscle cells, all GLUT4 molecules cycle to the PM (Foster *et al*, 2001), yet only 50% of GLUT4 is sensitive to TfR-mediated ablation (V.K. Randhawa, T.E. McGraw & A. Klip, unpublished data). The concept that GLUT4 can follow two distinct exit routes towards the PM is buttressed by the participation of VAMP2 in insulin-dependent, but not constitutive, GLUT4 recycling (Cheatham *et al*, 1996; Olson *et al*, 1997; Martin *et al*, 1998; Randhawa *et al*, 2004).

Model 2, championed by James and colleagues, proposes that only a fraction of GLUT4 recycles to the PM in the basal state, that insulin increases the quantity of GLUT4 available for translocation, and that part of the TfR-negative GLUT4 compartment interfaces with the TGN (Coster *et al*, 2004). Therefore, increasing insulin doses progressively engages a larger fraction of the non-cycling pool, which is then mobilized towards the PM through a single exit route—that is, from the ERC. Intriguingly, a component of the non-cycling pool contains 'latent GLUT4 molecules' that are not mobilized, even in response to insulin. It is speculated that the latent pool contains transporters that are synthesized early in cellular life and are preferentially excluded from the insulin response (Govers *et al*, 2004). The TGN participation in GLUT4 storage is inferred from the partial colocalization of GLUT4 and IRAP with the mannose 6-phosphate receptor, the adaptor-related protein complex-1 (Bryant *et al*, 2002), syntaxin6 and syntaxin16, and with the acquisition of sialic acid during the recycling of IRAP treated with neuraminidase while at the cell surface (Shewan *et al*, 2003). Notably, however, there was no colocalization of GLUT4 with TGN38, which is an established TGN marker. The TGN has heterogeneous functional domains and, hence, whether it interfaces with the SC/GSV remains an open question.

Models 1 and 2 differ in the extent to which cellular GLUT4 recycles in the basal state, in the presence of a static/latent pool of GLUT4, in the contribution of the TGN as a storage site for GLUT4 and in the number of insulin-dependent GLUT4 exit routes towards the PM. Model 1 rests on studies performed in 3T3-L1 adipocytes transiently expressing haemagglutinin (HA)–GLUT4–enhanced green fluorescent protein (eGFP) or L6 muscle cells stably expressing GLUT4myc. Conversely, Model 2 arises from studies in 3T3-L1 adipocytes stably expressing HA–GLUT4. The observed discrepancies are, therefore, not likely to be caused by differences between stable or transient expression of the transporter, and the possible contribution of cell clonal background or GLUT4-expression levels must instead be considered.

Insulin signals directing GLUT4 cycling

The intricacies of insulin signalling have been reviewed recently (Gual *et al*, 2005; Thong *et al*, 2005). Insulin binding to, and activation of, its receptor is rapidly followed by docking of insulin-receptor substrates and activation of Class IA phosphatidylinositol 3-kinase (PI3K). The latter catalyses the formation of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P_3), which, in turn, leads to the activation of atypical protein kinase C (aPKC)λ/ζ and

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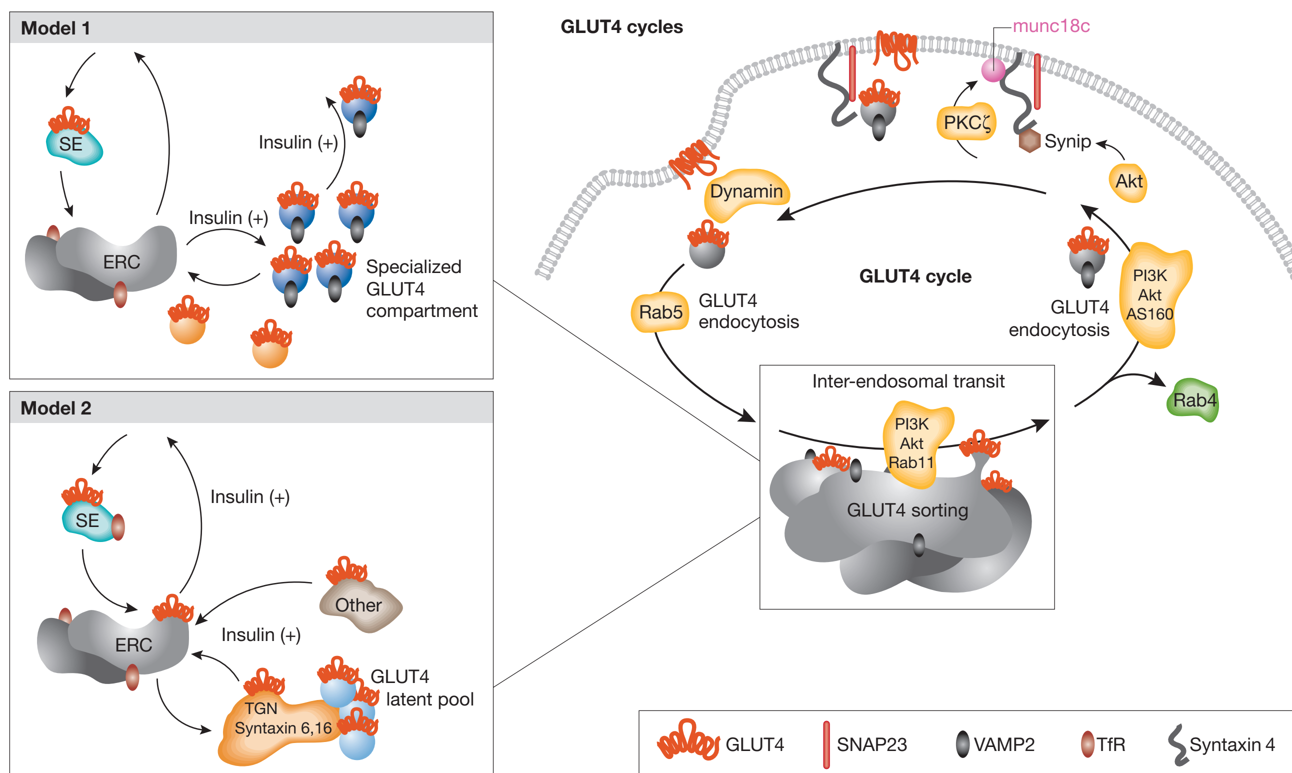

Fig 1 | Models of insulin-responsive glucose transporter 4 (GLUT4) compartments and cycling. Illustrated is a hypothetical version of GLUT4 cycling summarizing the current literature. GLUT4 cycles between intracellular compartments and the plasma membrane (PM) by the coordinated regulation of exocytic mobilization, fusion with the PM, endocytosis, and inter-endosomal transit and sorting. Each stage of GLUT4 transport is regulated by insulin-derived signals. The contents of the expanded box illustrate aspects of the two debated models of GLUT4 compartments and exit modalities. The models differ in negating or supporting the presence of a static/latent pool of GLUT4, in the contribution of the *trans*-Golgi network (TGN) as a storage site for GLUT4, and in the number of GLUT4 exit routes towards the PM. The plus symbol (+) indicates stimulation. AS160, Akt-substrate of 160 kDa; ERC, endosomal recycling compartment; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; SE, sorting syndrome; TfR, transferrin receptor.

Akt/PKB, enzymes required for insulin-induced GLUT4 translocation (Farese *et al*, 2005; Welsh *et al*, 2005). Akt/PKB acts on more than 35 substrates that are involved in various metabolic and mitogenic processes. Of these, the Rab–GTPase-activating protein (GAP) Akt substrate of 160-kDa (AS160) participates in GLUT4 translocation to the PM (Sano *et al*, 2003), presumably through its recently identified targets Rab2, Rab8, Rab10 and/or Rab14 (Miinea *et al*, 2005). Supporting these observations, Rab10, Rab11 and Rab14 were found on GLUT4 vesicles, and silencing of the *AS160* gene by small-hairpin RNAs (shRNAs) elevated the insulin-dependent increase in surface levels of GLUT4 (Larance *et al*, 2005). Bolstering these studies, it was recently shown that knockdown of AS160 in unstimulated adipocytes increases surface levels of GLUT4 and glucose uptake, suggesting that AS160 contributes to GLUT4 retention in the basal state (Eguez *et al*, 2005). Additionally, a PI3K-independent pathway involving c-Cbl associated protein (CAP), Cbl and the GTPase TC10 could regulate GLUT4 translocation (Saltiel & Pessin, 2002), but apparently this is specific to adipocytes (JeBailey *et al*, 2004). We next focus on the input of insulin-derived signals in several discrete steps of the GLUT4 cycle: exocytic mobilization, fusion with the PM, and internalization and intracellular sorting (Fig 1).

GLUT4 exocytic mobilization. Insulin increases the rate of GLUT4 exocytosis in a PI3K-dependent manner (Yang *et al*, 1996). Accordingly, delivery of PI(3,4,5)P_3 increases surface GLUT4 levels, whereas PI3P (the product of Class II and Class III PI3K) facilitates the arrival of GLUT4 vesicles at the PM without allowing fusion (Maffucci *et al*, 2003; Ishiki *et al*, 2005; Kanda *et al*, 2005). Therefore, distinct phosphoinositides regulate the arrival and fusion of GLUT4 vesicles with the PM. Insulin-activated PI3K also results in the activation of the Rho-family protein Rac, which, in turn, leads to actin remodelling beneath the membrane of muscle cells (JeBailey *et al*, 2004). A PI(3,4,5)P_3-independent input similarly activates the GTPase TC10 to reorganize actin in adipocytes (Saltiel & Pessin, 2002). Remodelled actin sites could contribute to GLUT4 mobilization by promoting GLUT4 and signal sorting, guiding myosin motors on GLUT4 vesicles or positioning GLUT4 near fusion sites on the PM (Patel *et al*, 2005). Insulin-induced actin remodelling does not require Akt, but this enzyme (predominantly Akt2) is required for the net GLUT4 translocation process, as illustrated by the use of dominant-negative mutants, gene silencing via RNAi and *Akt2*-gene knockout (Welsh *et al*, 2005). Akt directly targets GLUT4-containing endomembranes, as constitutively active or inactive

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mutants of the enzyme targeted to GLUT4-containing compartments respectively emulate or reduce the insulin response of GLUT4/IRAP. Accordingly, AS160 has been implicated in the GLUT4-mobilization step on the basis of a reduction in GLUT4–eGFP signal beneath the PM induced by overexpression of a non-phosphorylatable mutant of AS160, as detected by total internal reflection fluorescence microscopy (Zeigerer *et al*, 2004). The aPKCs also affect GLUT4 mobilization towards the PM by promoting association between the GTPase Rab4, the motor protein KIF3 and the microtubules required for such mobilization (Imamura *et al*, 2003). In summary, these studies imply that the PI3K→Akt→AS160 and PI3K→aPKC axes regulate GLUT4 mobilization towards the PM. How individual, complementing or redundant these two axes are remains to be defined.

GLUT4-vesicle fusion with the PM. In addition to regulating GLUT4 vesicle mobilization, insulin-derived signals also impinge on their fusion with the PM. At 19 °C, insulin elicits GLUT4 mobilization towards the PM without causing Akt activation or GLUT4 fusion, which instead rapidly ensue on re-warming to 37 °C (van Dam *et al*, 2005). Akt can target components of the vesicle–PM fusion machinery, which comprises the vesicular VAMP2, the target membrane SNAP23 and syntaxin4 (SNAREs), as well as synip, tomosyn and munc18c that bind syntaxin4 to modulate the insulin-dependent gain in surface GLUT4 (Widberg *et al*, 2003; Hodgkinson *et al*, 2005). Akt-dependent synip phosphorylation liberates syntaxin4, presumably to promote SNARE-complex formation (Yamada *et al*, 2005); however, the need for synip phosphorylation in insulin-dependent GLUT4 translocation has been contested by mutagenesis studies (Sano *et al*, 2005). As in the case of Akt, aPKCs have an input in GLUT4 fusion. PKCζ seems to phosphorylate VAMP2 (Braiman *et al*, 2001), and insulin promotes the formation of a complex that includes PKCζ and munc18c, thereby dissociating munc18c from syntaxin4 (Hodgkinson *et al*, 2005). Collectively, these molecular events could underpin the recent observation that insulin slows down the constitutive rapid movement of GLUT4 beneath the PM, and promotes vesicle tethering and fusion to increase surface GLUT4 levels (Lizunov *et al*, 2005). The regulation of fusion has been recently emphasized by *in vitro* studies whereby only PM isolated from insulin–stimulated cells were able to bind purified membrane vesicles containing GLUT4 (Koumanov *et al*, 2005).

GLUT4 endocytosis and inter-endosomal transit. GLUT4 internalization from the cell surface is reduced on microinjection into adipocytes of dynamin or amphiphysin peptides (Volchuk *et al*, 1998), and by expression of dynamin (Al-Hasani *et al*, 1998; Kao *et al*, 1998) or caveolin-3 mutants (Cohen *et al*, 2003). These results indicate that GLUT4 internalizes through both clathrin-coated pits and caveolae. Insulin modestly reduces GLUT4 endocytosis, in part by inhibiting Rab5 activity (Huang *et al*, 2001). In muscle cells, internalized GLUT4 reaches the early endosome in 2 minutes and the ERC in 20 minutes. Insulin accelerates GLUT4 arrival to, and departure from, the ERC, which is a process that requires Akt (Foster *et al*, 2001) and PIKfyve (Berwick *et al*, 2004). Such acceleration might contribute to the increased exocytosis of GLUT4 observed in response to insulin. More molecular detail of intracellular GLUT4 sorting in insulin-stimulated cells is required to improve our understanding of the unique action of this hormone.

Concluding remarks and open questions

GLUT4 cycling is regulated at the levels of its exocytosis, fusion, endocytosis and inter-endosomal transit. Most studies have assessed the impact of interfering with specific signalling molecules on particular segments of GLUT4 traffic. However, for the most part, this approach does not establish the precise target of the insulin-derived signals. It is pressing to identify the steps that are the primary recipients of insulin-derived signals and to differentiate them from secondary responses. For example, an insulin-derived signal might act on GLUT4-vesicle fusion at the PM, secondarily backing up the exocytic arm of GLUT4 cycling. Another potentially related question concerns the location of the SC/GSV in muscle and fat cells: is it perinuclear or found in the cytosolic pool of small vesicles? In addition, mapping the entire signalling cascades from receptor to GLUT4 and the precise site-of-action of each insulin-derived signal will be paramount to designing therapeutic strategies to relieve insulin resistance. Finally, it is important to identify molecules that bind to GLUT4, thereby determining its location, retention, and release from endomembranes and the PM. Faithful tracking of GLUT4 molecules in space and time might still reveal unexpected nuances of GLUT4 cycling in live muscle and adipose cells.

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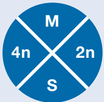

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