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Review

Mapping Insulin/GLUT4 Circuitry

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One of the most important metabolic actions of insulin is catalysing glucose uptake into skeletal muscle and adipose tissue. This is accomplished via activation of the phosphatidylinositol-3-kinase/Akt signalling pathway and subsequent translocation of GLUT4 from intracellular storage vesicles to the plasma membrane. As such, this represents an ideal system for studying the convergence of signal transduction and protein trafficking. The GLUT4 translocation process is complex, but can be dissected into at least four discrete trafficking steps. This raises the question as to which of these is the major regulated step in insulin-stimulated GLUT4 translocation. Numerous molecules have been reported to regulate GLUT4 trafficking. However, with the exception of TBC1D4, the molecular details of these distal signalling arms of the insulin signalling network and how they modify distinct steps of GLUT4 trafficking have not been established. We discuss the need to adopt a more global approach to expand and deepen our understanding of the molecular processes underpinning this system. Strategies that facilitate the generation of detailed models of the entire insulin signalling network will enable us to identify the critical nodes that control GLUT4 traffic and decipher emergent properties of the system that are not currently apparent.

Key words: Akt, GLUT4, insulin, signalling, TBC1D4, trafficking

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Mammals have evolved an exquisite homeostatic mechanism for maintaining a stable blood glucose concentration. In the fasting state, blood glucose is supplemented via hepatic glycogenolysis, where liver glycogen is broken down to glucose, or via gluconeogenesis, where 3C intermediates, such as lactate, are converted back to glucose in the liver. After a meal, the peptide hormone insulin is secreted from β-cells located in the islets of Langerhans in the pancreas. Insulin inhibits glucose output from the liver and promotes the uptake of glucose from the blood stream into skeletal muscle and adipose tissue. Insulin signals to a number of cellular processes such as protein synthesis, gene transcription and metabolism. The most significant effect of insulin on glucose metabolism is the regulation of GLUT4 trafficking and consequently glucose uptake. The reason for this is that although the intracellular pathways that determine the fate of glucose, such as glycolysis, glycogen synthesis and lipogenesis, are regulated by insulin, it is widely believed that glucose transport is rate limiting for most of these processes. Furthermore, the relative contribution of insulin-stimulated post-translational modifications and allosteric regulation by metabolites in controlling the activity of enzymes that regulate glucose metabolism remains unclear. Recent data have demonstrated that allosteric regulation of glycogen synthase (GS) by glucose-6-phosphate may be more important for regulation of its activity than phosphorylation by GSK-3β (1). Thus, glucose transport is the key step in insulin-regulated glucose metabolism and it is clear that defects in this process in muscle and adipose tissue represent a very early defect in the onset of insulin resistance (2). In this article, we will focus on the intersection between insulin signalling and GLUT4 trafficking. As such, we will devote a significant section of this review to critically analyse the current data implicating the Rab GTPase activating proteins, TBC1D4/AS160 and TBC1D1, in insulin-regulated GLUT4 trafficking. There have been a number of recent review articles discussing our current understanding of the insulin signalling pathway (3–5). We highlight the key questions that remain regarding the junctions between the insulin signalling network and GLUT4 trafficking pathway and propose that a global systems biology strategy is necessary to fully decipher how insulin regulates cellular processes.

The Insulin Signalling Pathway

In insulin-responsive tissues such as skeletal muscle, adipose tissue and liver, insulin signalling is initiated following binding of insulin to the α-subunit of the insulin receptor on the cell surface. This results in autophosphorylation of the β-subunit and activation of the receptor intrinsic tyrosine kinase activity (6). The active tyrosine kinase phosphorylates insulin-receptor substrate (IRS) proteins. Phosphorylated IRS proteins recruit phosphatidylinositol-3-kinase (PI3K) through the p85 regulatory subunit leading

to the activation of the catalytic subunit p110. PI3K catalyses the formation of phosphatidylinositol (3,4,5)P3 from phosphatidylinositol (4,5)P2 on the cytosolic leaflet of the plasma membrane (PM). The connector enhancer of KSR-1 protein (CNK1) was recently implicated in regulating insulin signalling through IRS-1 and PI3K by being part of a complex that indirectly stimulates the activity of phosphatidylinositol-4-phosphate 5-kinases at the PM. Depletion of CNK1 via siRNA resulted in loss of IRS-1 and Akt phosphorylation, presumably due to a reduction in PI(4,5)P2 at the PM, which are important for both the recruitment of IRS-1 to the PM via its PH domain and providing substrate for PI3K (7).

PI(3,4,5)P3 acts as a docking site for the serine/threonine kinase Akt, a member of the AGC kinase family. Following its recruitment to the cell surface, Akt is activated (as described below) resulting in the Akt-dependent phosphorylation of many substrates. In adipose and muscle, this results in the translocation of the GLUT4 glucose transporter from the intracellular storage compartment to the PM; enhancing glucose uptake into these tissues (reviewed in 8). In addition to glucose uptake, most, if not all, of insulin's metabolic effects are regulated by Akt. For example, Akt-dependent phosphorylation of GSK-3β leads to activation of glycogen synthase (GS) and enhanced glucose storage as glycogen. The classical insulin signalling cascade, comprising numerous cellular signalling events, is quite well understood. Despite this, additional components of the insulin signalling network are continuously being identified. Furthermore, there remains a lack of information on some aspects of insulin signalling, particularly on signalling events downstream of Akt that directly mediate GLUT4 trafficking to, and fusion with, the PM.

The Protein Kinase Akt

Activation of Akt is a critical step in the control of insulin signalling. Indeed, the central role of Akt was exemplified by a study reporting that specific activation of Akt, independently of any upstream signalling, is sufficient to drive GLUT4 storage vesicles (GSVs) to fuse with the PM of 3T3-L1 adipocytes to a similar extent as insulin stimulation (9). However, this artificial system may override the requirement for normal biological regulatory mechanisms. Active Akt, targets a series of downstream substrates that mediate the diverse cellular functions of the insulin signalling pathway. The challenge is to dissect the full repertoire of substrates and to link them to a biological function. As described below there is considerable evidence now to suggest that Akt regulates GLUT4 translocation to the PM. While some Akt substrates involved in this process have been identified, the complex nature of GLUT4 trafficking suggests that others remain to be discovered.

There are three isoforms of Akt in mammals; each shares the same domain organization and are encoded by separate genes sharing greater than 85% homology (10). Akt1 is ubiquitously expressed, while expression of Akt2 is highest in insulin-responsive tissues such as heart, liver, kidney and skeletal muscle (11). The expression of Akt3 is limited to the brain and testes (12). The varied expression profile of the different isoforms raised the possibility that each isoform plays a unique role in tissue-specific signalling. Several studies in knock-out mice missing a single Akt gene have supported this hypothesis, with only deletion of Akt2 resulting in a specific defect in glucose metabolism (13). Consistent with these data, Akt2 has been reported to be preferentially recruited to the PM in response to an insulin stimulus (14). The different isoforms of Akt are thus best described as having distinct cellular functions while displaying a degree of redundancy between isoforms. Furthermore, there is a nonlinear relationship between Akt activation and physiological output; the activation of only a small proportion of total cellular Akt is sufficient for a maximal physiological response (15).

Activation of Akt

The relocalization of Akt to the PM from the cytosol is crucial for its activation; Akt mutants that are constitutively targeted to the PM are constitutively active (16). Binding of PI(3,4,5)P3 to the PH domain induces a conformational change in Akt, which is thought to be a necessary step prior to its phosphorylation at Thr^{308} by 3-phosphoinositide dependent protein kinase-1 (PDK-1) (17–19). PDK-1 appears to be constitutively active, and is found both at the PM and in the cytosol, implying that it is not a regulated node in the control of Akt activity (20,21). An intramolecular interaction between the PH and kinase domains of Akt has been suggested to prevent phosphorylation of Thr^{308} by PDK-1 and thus activation. This repression is relieved by binding of PI(3,4,5)P3 by the PH domain, inducing a conformational change in Akt and allowing activation via PDK-1 (19). A recent study using Förster resonance energy transfer suggested that Akt binds PDK-1 in the cytosol, and this complex translocates to the PM after growth factor stimulation (19). This pre-activation complex may allow an extremely rapid activation of Akt upon translocation to the PM. Phosphorylation of Thr^{308} by PDK-1 causes a conformational change in the activation loop, causing it to 'flip out' of the active site allowing binding of both ATP and the substrate protein.

A second regulatory phosphorylation site, Ser^{473}, is present in the C-terminal hydrophobic motif of Akt. The search for the kinase that phosphorylates Ser^{473}, the so-called 'PDK-2' has been long and controversial. Various candidate molecules have been proposed, including protein kinase Cα (PKCα), the integrin-linked kinase, ATM, DNA-PK and autophosphorylation by Akt itself (reviewed in 22). However, the mammalian target of rapamycin (mTOR) in complex with mLST8, mSin and rictor (the mTORC2 complex) has emerged as a strong candidate for the Ser^{473} kinase. Sarbassov et al. (23) reported that the mTORC2 complex is necessary for Ser^{473} phosphorylation and directly phosphorylates Akt at Ser^{473}. In addition, mTORC2 was shown to be the sole kinase responsible for

Ser^{473} phosphorylation in the 3T3-L1 cell line (24). Tissue-specific knock-out of rictor in skeletal muscle and adipose tissue resulted in decreased insulin-stimulated glucose uptake and GLUT4 translocation (25,26). Phosphorylation of Ser^{473} has been proposed to play a dual role in the activation of Akt, acting as a docking site for PDK-1 in other AGC family kinases (27) and as an allosteric regulator of Akt activity. However, it has also been reported that phosphorylation of Thr^{308} can occur before phosphorylation of Ser^{473} (28). The requirement for Ser^{473} phosphorylation prior to that of Thr^{308} has also been challenged by experiments using an animal knock-out of Sin1, a component of the mTORC2 complex. Despite loss of Ser^{473} phosphorylation, phosphorylation at Thr^{308} was maintained both in knock-out mice and derived Mouse embryonic fibroblasts (MEFs) (29). Additionally, tissue-specific knock-out of rictor does not result in impaired phosphorylation of all Akt substrates. For example, phosphorylation of GSK-3β at Ser^{9} remains unaffected (25,26).

The activation of Akt's kinase activity therefore requires three steps: translocation to the PM and binding of PI(3,4,5)P3, subsequent phosphorylation of Thr^{308} by PDK-1 and Ser^{473} probably by the mTORC2 complex. However, the order of these phosphorylation events is not yet clear, and the spatial regulation of the interaction with PDK-1 remains contentious. Additionally, active Akt must translocate to different subcellular compartments to phosphorylate its many substrates (www.jameslab.com.au/Contentpages/DataResources/KnownAktSubstrates.shtml). Recent publications have begun to describe signalling pathways that may play a role in controlling the activity of Akt and therefore provide for additional modulation of insulin signalling. The pleckstrin homology-like domain family B member 1 protein (PHLDB1) translocates to the PM in response to an insulin stimulus. Depletion of PHLDB1 via siRNA reduced Akt phosphorylation, glucose uptake and GLUT4 translocation in 3T3-L1 cells, although the mechanism by which this occurs remains unclear (30). What is especially interesting is that PHLDB1 is itself predicted to be an Akt substrate, implying a tight feedback loop. Akt has also been reported to undergo protein tyrosine kinase 6-dependent phosphorylation in its catalytic domain, enhancing Akt activation in response to a growth factor stimulus in an *in vitro* prostate cancer model (31).

The substrate specificity of Akt also appears to be linked to its spatial distribution. Akt has been reported to localize to different domains within the cell including the PM, endosomes and GSVs. The regulation of its distribution is likely to be regulated by both lipids and scaffolding proteins. Appl1 targets Akt to endosomes (32) and the protein scaffold CNK1, in addition to indirectly mediating the activation of Akt as described above, can also directly bind to Akt (33). Further examination of Akt-binding partners may shed further light on the features of Akt action such as how Akt spatial distribution is achieved, whether protein scaffolds can regulate Akt activity and where Akt interacts with its substrates.

Signalling Events that Control GLUT4 Trafficking

There are a number of trafficking events in the intracellular itinerary of GLUT4 which may be targeted by insulin signalling to enhance GLUT4 PM levels. This includes, the release of GSVs from intracellular retention, GSV translocation to the cell surface (which likely involves transport of GSVs along cytoskeletal structures), tethering, docking and fusion of GSVs at the PM and finally endocytosis or removal of GLUT4 from the cell surface (reviewed in 34) (Figure 1). Recent work has uncovered novel insulin-mediated signalling events at many of these steps.

Signalling events leading to the release of GLUT4 vesicles from the storage compartment

There is significant debate as to whether GLUT4 is retained intracellularly in basal conditions by a static or dynamic retention mechanism (35,36). These models differ not only in their mechanism for GSV retention, but also in the manner in which GLUT4 is delivered to the membrane, with static retention favouring a quantal release mechanism and dynamic retention implying a continuous-flow release mechanism. Regardless, the release of GSVs from an intracellular site for fusion with the PM is likely the most significant site of insulin-stimulated alterations in GLUT4 trafficking. The most intuitive mechanism by which GLUT4-containing vesicles could be sequestered is by tethering to protein scaffolds. TUG and p115 have been reported to interact with distinct GSV-resident proteins and disruption of their function by over-expression of a truncated form of these proteins induces a phenotype indicative of a role in tethering GSVs (37,38). However, there is currently no published data indicating that these tether proteins might be a site of signalling in insulin-induced GLUT4 translocation.

A breakthrough in determining how the insulin signalling pathway distal to Akt activation may signal to release GSVs from retention came in 2002, with the discovery of the Akt substrate of 160 kDa (AS160, also known as TBC1D4). TBC1D4 was first identified in insulin-stimulated 3T3-L1 adipocytes using an antibody specific for the Akt phosphorylation motif (RxxRxS/T) (39). This finding was particularly exciting as TBC1D4 is a Rab GTPase activating protein (Rab-GAP) and therefore offered a connection between insulin signalling through Akt and membrane trafficking events leading to GLUT4 translocation. There is now a plethora of experimental data from investigations utilizing various tools and techniques to identify the exact role of TBC1D4 in insulin-regulated GLUT4 trafficking (reviewed in 3). These approaches have included siRNA to knock-down TBC1D4 expression levels, a dominant mutant in which four insulin-sensitive phosphorylation sites (S318A, S588A, T642A and T751A) were mutated to alanine (TBC1D4-4P), site-specific antibodies to interrogate phosphorylation at distinct sites within TBC1D4 and the use of 14-3-3-binding mutants to investigate the role of 14-3-3 binding in the regulation of TBC1D4 activity. These

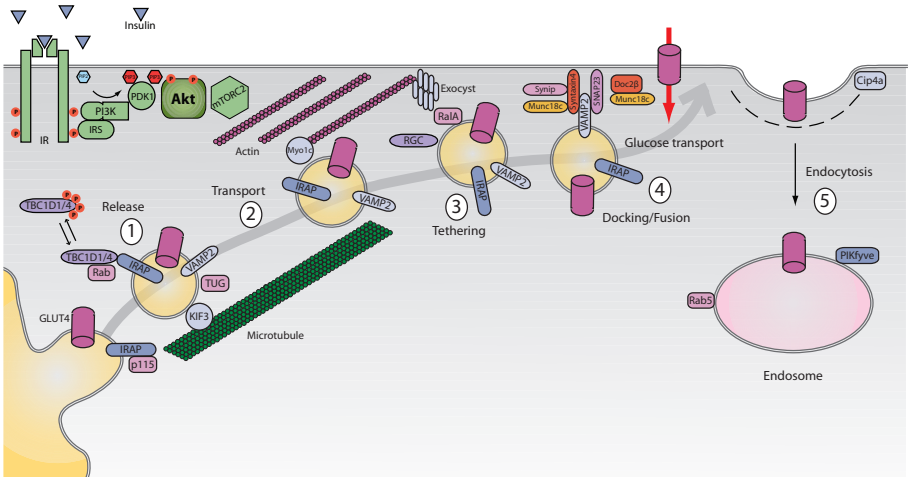

Figure 1: Overview of molecules that are involved in insulin-regulated GLUT4 trafficking as described in this review. The binding of insulin to its receptor triggers an intracellular signalling cascade that culminates in the recruitment of Akt to the PM where it is activated by phosphorylation. Through numerous downstream substrates, insulin signalling intersects the GLUT4 trafficking pathway at multiple steps including: the release of GSVs from retention (step 1), trafficking of GSVs to the PM via the cytoskeleton (step 2), GSV tethering (step 3), docking and fusion with the PM (step 4) and GLUT4 internalization from the cell surface (step 5).

data have led to the hypothesis that TBC1D4 retains GLUT4 within the cell by inactivating its cognate Rab protein. Phosphorylation of TBC1D4 by Akt in response to an insulin stimulus recruits 14-3-3, which binds to TBC1D4. These signalling events inhibit TBC1D4 GAP activity, allowing GTP loading and activation of the Rab protein. This would increase the concentration of GTP-Rab on GSVs, ultimately resulting in their translocation to, and fusion with, the PM.

Interestingly, the hypothesis described above represents only an incremental advance when compared to the initial hypothesis put forward following the discovery of TBC1D4. Indeed, despite the large amount of data now published on TBC1D4 interacting partners, phosphorylation status and 14-3-3 binding, a number of questions remain unanswered: What is the mechanism for inactivation of TBC1D4 GAP activity? Which Rab or Rabs does TBC1D4 inactivate? Does TBC1D4 translocate from GSVs to the cytosol upon insulin stimulation? What is the precise role of 14-3-3 binding? Where in the cell is TBC1D4 phosphorylated? Which step or steps in GLUT4 trafficking does TBC1D4 regulate? Is TBC1D4 the predominant mediator of insulin's effect on GLUT4 trafficking?

We propose that the techniques currently in use may not provide the entire story regarding the role and importance of TBD1D4 in GLUT4 translocation. For example, siRNA knock-down of TBC1D4 results in an expected yet moderate phenotype. Adipocytes with reduced expression of TBC1D4 have increased GLUT4 levels at the PM in the basal state, consistent with TBC1D4 having a negative role in controlling GLUT4 exocytosis (40). However, knock-down of TBC1D4 does not phenocopy insulin; approximately two-thirds less GLUT4 is redistributed to the PM than following insulin stimulation. This suggests that there is a minimum amount of TBC1D4 that is sufficient to retain GLUT4 intracellularly. It is possible that there is a degree of 'spareness' in the signalling network at the level of TBC1D4. This hypothesis is supported by recent data from our laboratory indicating that only a small proportion of total cellular TBC1D4 needs to be phosphorylated in order for insulin to induce maximal GLUT4 translocation (41). An alternative explanation is that other TBC1D4-independent processes act either to retain GLUT4 intracellularly or promote GSV fusion with the PM.

Finally, it is possible that TBC1D4 itself has a secondary function at the cell periphery. This hypothesis is supported by two lines of evidence. First, TBC1D4 knock-down results in reduced insulin-stimulated GLUT4 exocytosis. This argues for an additional, positive function for TBC1D4 distal to GSV sequestration (40). Second, the use of TBC1D4 phosphorylation mutants in combination with microscopic techniques, such as total internal reflection fluorescence (TIRF), has revealed a role for TBC1D4 in GSV docking and fusion (42,43). However, other studies have reported that the primary defect in cells expressing these mutants is at the level of GSV release from sequestration (44). This confusion may stem from differences in the expression level and thus efficacy of the mutant protein. For example, in studies with high over-expression of TBC1D4 phosphorylation mutants, GLUT4 release from sequestration may be completely inhibited. In this case the strong inhibition at one step (GSV release) prevents the measurement of an effect of this mutant downstream (at the PM). Therefore, the data from experiments carried out under these conditions does not preclude the possibility that TBC1D4 also acts at other sites in the cell to regulate GLUT4 trafficking. Performing

the same experiment in cells over-expressing the TBC1D4 mutant to a lesser degree might enable examination of the role of TBC1D4 at the PM.

It is of interest that despite nearly a decade passing since the discovery of TBC1D4, a knock-out mouse model has yet to be described. Recently, a knock-in mouse model in which the Thr^{642} phosphorylation and 14-3-3-binding site has been mutated to alanine has been reported (45). As expected, these mice are glucose intolerant and insulin insensitive. However, although these mice display defective glucose uptake into muscle, there was no defect in insulin-stimulated glucose uptake into adipose tissue. Therefore, although the whole-body phenotype of the TBC1D4 knock-in is entirely consistent with the current hypothesis for TBC1D4 action and importance, the tissue-specific molecular details of the phenotype are not clear.

A criticism of these genetic approaches (knock-in, over-expression) is that their chronic nature allows for compensatory mechanisms or non-specific interactions, in the case of over-expression, to occur. Just as the specific Akt inhibitors have greatly aided our understanding of isoform specificity for this kinase, a similar rapid method for targeting TBC1D4, either pharmacologically or through the rapid and reversible inducible expression of mutant proteins, would be of great benefit to the field.

TBC1D1, the closest homologue of TBC1D4, has an identical domain organization to TBC1D4. This Rab-GAP has also been implicated in regulating GLUT4 translocation (46). A mutation of TBC1D1 (R125W) has been linked to increased risk of familial obesity (47,48). Over-expression of TBC1D1, containing the R125W mutation, in muscle inhibited insulin-stimulated glucose transport (49). Additionally, insulin stimulation leads to increased phosphorylation of TBC1D1 at a site, which is predicted to be phosphorylated by Akt (Thr^{596}). Intriguingly, TBC1D1 and TBC1D4 have mutually exclusive tissue-expression profiles, raising the possibility that these Rab-GAPs may be functionally redundant. Congruent with this hypothesis, *Drosophila melanogaster* has only one TBC1D1/4 homologue, pollux which presumably fulfils the nutrient requirements of muscle in the fly in response to either feeding or exercise. In mammals, evidence is now building that TBC1D4 and TBC1D1 may play complementary rather than redundant roles. TBC1D1 has been linked with 5′ AMP-activated protein kinase (AMPK) and exercise/contraction-stimulated glucose transport, rather than insulin-stimulated glucose transport (50,51). Indeed, these proteins may act distinctly to regulate insulin (TBC1D4) or exercise/contraction (TBC1D1) stimulated glucose transport; perhaps offering an explanation for the additivity in stimulation of glucose transport and GLUT4 translocation observed between these signalling pathways (52,53). Another intriguing possibility is that TBC1D4 and TBC1D1 act as a site of cross-talk between the insulin and exercise/contraction signalling pathways. It has been reported that Akt can phosphorylate TBC1D1, and AMPK can phosphorylate TBC1D4 (54–56). As some tissues (e.g. plantaris and Extensor digitorum longus (EDL) muscles) express a significant amount of both isoforms (51), it would be advantageous for TBC1D1 and TBC1D4 to work synergistically in such a setting.

Complementarity between TBC1D4 and TBC1D1 can also be rationalized when considering the types of tissue with high TBC1D4 or TBC1D1 expression. For example, adipocytes, whose primary role is to dispose glucose in response to insulin, predominantly express TBC1D4. In contrast, glycolytic (white) muscle tissue, preferentially express TBC1D1; perhaps to respond primarily to high demand for glucose during exercise. It is clear that significant questions remain over the exact relationship between these two proteins. This problem is exacerbated by inconsistencies in both the organism and muscle types used for investigations into the role of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose uptake. It might be predicted that the phenotype of TBC1D1/4 disruption would depend on the fibre composition of the muscle studied.

Several Rabs have been proposed as the target of TBC1D4, although the identity of its target Rab(s) is controversial and remains an area of active research. Several Rab proteins have been identified as co-immunoprecipitating with GSVs in both 3T3-L1 adipocyte (57,58) and cardiac muscle cells (59). One of these, Rab10, has emerged as an attractive candidate for the target of TBC1D4 in 3T3-L1 cells; knock-down of Rab10 resulted in a reduction in insulin-stimulated GSV translocation to the PM and simultaneous knock-down of both Rab10 and TBC1D4 partially rescued the constitutive transport of GSVs to the PM caused by depletion of TBC1D4 (60). Conversely, in L6 muscle cells Rab8A, Rab13 (61) and Rab14 have been proposed as the major target Rabs of TBC1D4. Over-expression of Rab8A, Rab13 and Rab14, but not Rab10, reversed the constitutive block in GSV translocation caused by simultaneous over-expression of TBC1D4-4P in L6 cells (61,62). This suggests that there may be Rab degeneracy or cell-specific roles for these proteins in GLUT4 trafficking.

The apparent involvement of several different Rab proteins in GLUT4 trafficking may also reflect the complicated life cycle of a GSV. GLUT4 is recycled through several compartments before being packaged into specialized storage vesicles. Therefore, analysis of GLUT4-containing membranes that have been isolated by immunoprecipitation protocols will likely identify a multitude of Rab proteins that reflect this complicated trafficking route; it is reasonable to assume that these distinct GLUT4 trafficking steps are each controlled by a different Rab protein. In addition, identifying the Rabs that regulate discrete steps in GLUT4 trafficking is complicated as it requires assays to accurately measure specific and localized changes in GLUT4 trafficking. Intervening at any point in GLUT4 trafficking through disruption of Rab function may yield a phenotype

that can be misinterpreted if an end-point measurement such as the presence of GLUT4 at the cell surface is used.

Signalling to the cytoskeleton in GLUT4 vesicle trafficking

GLUT4 vesicles have been reported to associate with both the microtubule and actin cytoskeleton (63). Insulin signalling to the microtubule network appears to be mediated via motor proteins. For example, KIF3/kinesin II has been implicated in GLUT4 exocytosis. The association of KIF3/kinesin II with microtubules is enhanced via a PKCλ-dependent mechanism following insulin stimulation, although the details of this signalling mechanism remain unresolved (64).

A role for actin in mediating insulin-stimulated GLUT4 translocation has also been described. Insulin stimulation induces rapid remodelling of actin filaments into a cortical mesh in muscle cells and adipocytes (65). If this mesh formation is inhibited by administration of excess PI(3,4,5)P3 (66) or by the G-actin polymerization inhibitor latrunculin-B (65), insulin-induced GLUT4 translocation is inhibited. Interestingly, studies using TIRF have noted that disruption of cortical actin by latrunculin-B resulted in a normal redistribution of GLUT4 to the evanescent field (close to the PM), but inhibited GSV fusion with the PM. Several models have been proposed to explain actin's role in GLUT4 trafficking. Remodelled actin could facilitate GLUT4 translocation by localizing signalling intermediates, by promoting GLUT4 sorting, by guiding myosin motors on the GSVs or by positioning GLUT4 near the PM and aiding GSV docking with the PM. For this final model, an adaptor protein would likely be required to tether GLUT4-containing vesicles to cortical actin filaments. α-Actinin4 may provide this link (67).

Rho GTPases, such as Rac (43,68,69), which are known to mediate actin re-organization, have been reported to be activated by insulin signalling. However, the details of this activation are yet to be established. A more direct link between insulin signalling and the cytoskeletal may involve Myo1c, a motor protein that mediates movement along actin filaments. This protein was recently reported to be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II following insulin stimulation (70). Disruption of Myo1c function via siRNA or expression of an ATPase null mutant inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes and skeletal muscle, respectively (71,72). As Myo1c resides at the PM, it is likely function with actin at this location. The role of Myo1c is unclear, but it may act to regulate the formation of a protein complex or participate in the movement of GSVs along actin filaments towards the PM.

Signalling events at the PM; association and fusion of GSVs with the PM

Akt substrates have also been identified in the final steps of GLUT4 translocation; tethering of GLUT4-containing transport vesicles with the PM and membrane fusion. The exocyst complex, an evolutionarily conserved octameric protein complex, has been proposed to tether GLUT4 vesicles to the PM prior to fusion (73). Signalling to the exocyst complex is thought to occur through the small GTPase RalA, which is present on GSVs (74). Akt has been implicated in the control of RalA activity via phosphorylation of a dimeric Ral GAP protein complex (75).

The GSV fusion event is mediated by the action of the SNARE proteins Syntaxin 4, SNAP-23 and VAMP2/Synaptobrevin in concert with regulatory molecules such as Munc18c (76). The interaction between VAMP2 and Syntaxin 4 is regulated by a number of factors, one of which is the protein Synip, an Akt substrate. Phosphorylation of Synip in response to an insulin stimulus dissociates it from Syntaxin 4 and allows VAMP2 binding and GSV fusion with the PM (77). However, the involvement of Synip and its phosphorylation in regulating GLUT4 trafficking remains controversial (78). Insulin-stimulated phosphorylation of Munc18c and VAMP2 has also been reported. Munc18c is a regulator of SNARE complex formation and has been reported to undergo insulin-dependent tyrosine phosphorylation, switching its binding specificity from Syntaxin 4 to Doc2β (79), a calcium-sensitive positive regulator of exocytosis, which has been reported to be essential for triggering GSV fusion (80).

These recent discoveries of protein phosphorylation and interactions at the PM, downstream of insulin signalling are yet to be incorporated into a comprehensive model that definitively describes the complicated molecular details of GSV docking and fusion at the PM and how insulin signalling regulates these processes.

Signalling and GLUT4 endocytosis

GLUT4 is endocytosed via the canonical clathrin-dependent pathway (81). More recently, it has been reported that GLUT4 is endocytosed via multiple pathways in 3T3-L1 and L6 cells (82,83). GLUT4 possesses several trafficking motifs including an FQQI motif in its cytosolic N-terminus and a dileucine motif in its C-terminus that appear to function in endocytosis of the transporter. It has also been reported that endocytosis plays a role in the regulated accumulation of GLUT4 at the PM in response to insulin in both 3T3-L1 adipocytes and L6 myotubes (53,82,84). This effect is blocked by wortmannin, a PI-3-kinase inhibitor, but not by a specific Akt inhibitor; implying that inhibition of GLUT4 endocytosis in insulin-stimulated cells is an Akt-independent process (53,85). The Cdc42-interacting protein-4 was recently described, perhaps counterintuitively, to enhance GLUT4 internalization in insulin-stimulated L6 myoblasts (86). Rab5 has also been implicated in regulated GLUT4 endocytosis; Rab5 activity is inhibited following insulin stimulation (87). This could explain reduced GLUT4 endocytosis in insulin-stimulated cells as Rab5 is known to be involved in directing vesicle transport and endosomal fusion in the

early endocytic pathway. Further effort is required to pinpoint the role of Rab5 and other intermediates in insulin regulation of GLUT4 endocytosis. As the primary site of action for proteins regulating endocytosis is likely to be the PM, a targeted approach measuring signalling events [protein–protein interactions, post-translational modifications (PTMs)] specifically at this location may reveal the mechanism by which GLUT4 internalization is controlled.

Once internalized from the PM, GLUT4 is sorted back into its storage compartment via the endosomal system. This step may also be regulated by insulin action. The lipid kinase PIKfyve (*P*hospho*I*nositide *K*inase for five position containing an FYVE finger), a protein is thought to regulate endosomal trafficking, has been identified as an Akt substrate. Ablation of the Akt phosphorylation site by mutation to alanine in the PI(3)P 5-kinase PIKfyve enhances insulin-stimulated translocation of GLUT4 to the PM (88). However, the exact site of PIKfyve action and its role in GLUT4-regulated traffic remains unresolved.

Future Prospects: Proteomics and Functional Analysis of Integrated Signalling Networks

To date, the majority of our knowledge of the insulin signalling cascade is derived from reductionist investigations focussing on individual proteins or processes. As described in this article, this has led to the identification of the canonical PI3K/Akt pathway, Akt substrates and some functional links. However, our view of this pathway is still somewhat rudimentary. We view it as a linear analogue pathway, which tends to overlook important topological features.

We propose that there are three issues that, if addressed, will enable a more comprehensive understanding of the complex network of signalling elements that lead to GLUT4 trafficking following insulin stimulation. First, the multitude of insulin signalling features such as PTMs and protein–protein interaction need to be identified. Second, a specific function needs to be assigned to these events. Finally, these data should be modelled using *in silico* dynamical approaches so that the insulin signalling network leading to GLUT4 translocation can be viewed as a whole and novel features of the pathway, such as feedback loops, revealed.

Mass spectrometry-based protein identification has led to several important advances in the field, none more so than the identification of TBC1D4 as an Akt substrate (39). However, over the past few years, there have been considerable advances in mass spectrometry methodologies and technologies. These include both improvements in instrumentation to enhance mass accuracy and mass spectra acquisition speed and algorithms for peptide identification and PTM assignment. This has been coincident with the rise of techniques that permit accurate relative and absolute quantification of protein by mass spectrometry [e.g. stable isotope labelling in cell culture (SILAC) and iTRAQ]. These improvements have resulted in a shift in the focus of mass spectrometry-based experiments from protein identification to more functional analysis. This change in application is made all the more exciting by the fact that proteomic laboratories can now routinely and reliably identify and quantify thousands of proteins and PTMs per experiment.

The small number of studies that have taken advantage of these proteomic techniques to study features of the insulin signalling pathway to date highlight the success that the combination of high-resolution mass spectrometry with differential mass labelling (e.g. SILAC or iTRAQ) can have when applied to this signalling network. Using various chromatographic enrichment strategies, these studies focussed on identifying and quantifying specific insulin-responsive PTMs. In each case, the authors successfully identified novel insulin-sensitive tyrosine phosphorylated proteins and Akt substrates which, in some cases, represent cellular processes that were previously not known to be regulated by insulin (89–92).

It is clear that in-depth proteomic analysis of insulin signalling will yield additional components of this signalling network and further our understanding of insulin action. This will not only lead to the discovery and cataloguing of PTMs across the proteome, but will also allow these PTMs to be studied in a more functional manner. This is exemplified by investigations using a phosphotyrosine-targeted approach. Here, the authors provided detailed kinetic information on the tyrosine phosphorylation of substrates in response to insulin, and identified interacting partners associated with specific phosphorylation events on the insulin receptor and IRS proteins (90,91). As tyrosine phosphorylation only represents a very small proportion of total cellular phosphorylation, these phosphoproteomic and interactomic analyses will need to be extended to include serine/threonine phosphorylation. These approaches can also be used to interrogate signalling processes at specific locations within the cell; a focus on the PM may reveal the mechanism by which insulin signalling controls the features of GLUT4 trafficking that occur at the cell periphery.

The multitude of signalling events within the insulin signalling pathway that will be identified by such a proteomic approach must be further investigated in order to determine their specific role in regulated GLUT4 translocation. This is not a trivial task when considering a complex process such as GLUT4 trafficking. Ideally, assays that can distinguish between different trafficking steps such as GLUT4 trafficking to the PM, docking with the PM, GSV fusion and GLUT4 endocytosis will be used to accurately assign roles to signalling interventions. Simply measuring the effect of distinct signalling intermediates on an end-point measurement, such as GLUT4 appearance at the PM or glucose uptake, will not identify the

specific process or processes that these signalling intermediates regulate. Importantly, using techniques already adopted in trafficking studies (antibody uptake assays and internalization assays), TIRF and confocal microscopic techniques and an *in vitro* fusion assay (93), researchers have described assays that are able to monitor distinct steps of GLUT4 trafficking (35,43,44,94). The interaction of GSVs with the PM is a particularly complex yet important event in GLUT4 trafficking. Advances in analysis of GSV trafficking features, the formation of signalling complexes and protein–protein interactions by high-resolution TIRF microscopy at this location will enable a deeper understanding of signalling events at the PM.

As hinted at above, the true power of high-resolution proteomics and interactomics, in combination with functional assignment, lies in their analysis by sophisticated systems biology modelling. In particular, the advantage to a systems biology approach in the context of complex integrated signalling networks is that multiple parameters can be considered when modelling. This is vital as the insulin signalling pathway comprises elements that are regulated by a number of different mechanisms. This includes integrating temporal data on transient PTMs and protein–protein interactions, with details of allosteric regulation and information on spatial regulation of signalling complexes.

Some specific aspects of insulin signalling leading to GLUT4 translocation have been mathematically modelled (95,96). However, the recent advances in quantitative proteomics described above offer the opportunity to revisit these modelling concepts with hugely enhanced data sets. In addition, this modelling can be readily accessed by researchers without a specialized mathematical background as several computational tools have been generated to facilitate the generation signalling networks from data sets (reviewed in 97). In this way it will be possible for researchers to view the insulin signalling network to GLUT4 translocation and (other cellular processes) as an integrated map.

Network modelling may reveal features of the insulin signalling network that could not have been envisaged using traditional cell biology approaches. For example, the GLUT4 trafficking itinerary depends on several distinct regulated processes. When considering the individual processes in isolation, it is likely that several binary interactions combine to generate a single biological event. As well as defining the temporal and spatial requirements for these individual processes, the mapping strategy described above will also reveal interplay between molecules involved in regulating multiple processes. It is likely that early signalling events in insulin-stimulated GLUT4 trafficking will feed forward onto subsequent processes; similarly distal processes may play a counter regulatory role. Analysing the insulin signalling network in this way will enable us to identify the key nodes in insulin signalling to GLUT4 trafficking that are dysregulated in insulin resistant cells.

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