

REVIEW

Small G proteins in insulin action: Rab and Rho families at the crossroads of signal transduction and GLUT4 vesicle traffic

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Abstract

Insulin stimulates glucose uptake into muscle and adipose tissues through glucose transporter 4 (GLUT4). GLUT4 cycles between the intracellular compartments and the plasma membrane. GLUT4 traffic-regulating insulin signals are largely within the insulin receptor-insulin receptor substrate-phosphatidylinositol 3-kinase (IR-IRS-PI3K) axis. In muscle cells, insulin signal bifurcates downstream of the PI3K into one arm leading to the activation of the Ser/Thr kinases Akt and atypical protein kinase C, and another leading to the activation of Rho family protein Rac1 leading to actin remodelling. Activated Akt inactivates AS160, a GTPase-activating protein for Rab family small G proteins. Here we review the roles of Rab and Rho proteins, particularly Rab substrates of AS160 and Rac1, in insulin-stimulated GLUT4 traffic. We discuss: (1) how distinct steps in GLUT4 traffic may be regulated by discrete Rab proteins, and (2) the importance of Rac1 activation in insulin-induced actin remodelling in muscle cells, a key element for the net gain in surface GLUT4.

Keywords GLUT4 traffic, insulin, muscles, Rabs, Rhos, small GTPases.

Skeletal muscle is the major tissue for dietary glucose disposal. Insulin stimulates glucose uptake into muscle cells by increasing the amount of glucose transporter isoform 4 (GLUT4) at the cell surface. GLUT4 is a member of the SLC2 facilitative sugar transporter family, which consists of 13 members with diverse substrate specificities, tissue expression patterns and regulation by hormones (Wood & Trayhurn 2003). The insulin-sensitive GLUT4 is primarily expressed in muscle and adipose tissues, which respond to insulin by increasing glucose uptake. In these cells, GLUT4 exists in multiple subcellular compartments including the plasma membrane (PM), and the endosomal network. GLUT4 travels in vesicles between these compartments, and its traffic and subcellular localization are highly regulated by insulin. In insulin-resistant states, notably in type 2 diabetes, GLUT4 translocation is impaired (Koistinen & Zierath 2002). Therefore,

understanding the mechanism of GLUT4 traffic and its regulatory inputs is of fundamental importance.

Small G proteins are molecular switches that participate in multiple biological processes including signal transduction, membrane traffic and cytoskeleton organization. These processes all participate in intracellular GLUT4 traffic and there is considerable evidence for the involvement of small G proteins in this insulin action. Here, we summarize insulin signalling and GLUT4 translocation with specific emphasis on the small G proteins involved.

GLUT4 translocation

In muscle and fat cells, GLUT4 cycles between the intracellular compartments and the PM (Fig. 1). In the basal state, more than 90% of GLUT4 is sequestered in the intracellular compartments, and approximately

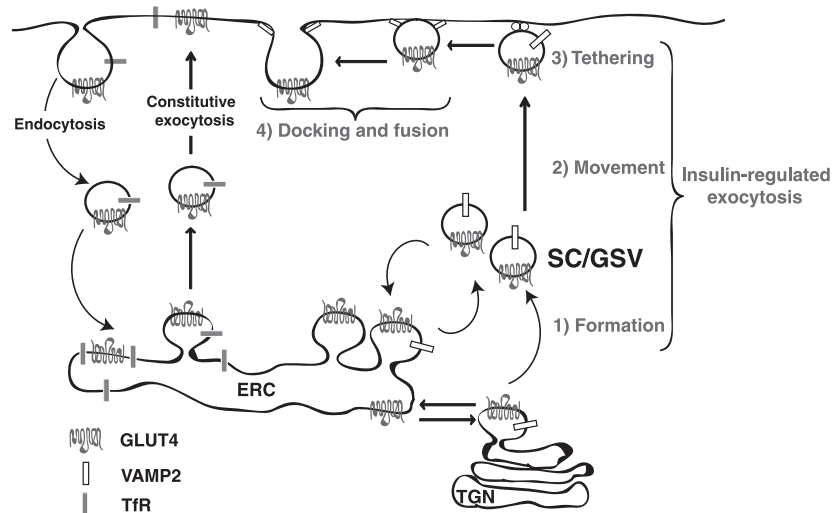


Figure 1 Schematic representation of glucose transporter 4 (GLUT4) traffic in muscle and fat cells. GLUT4 cycles between the intracellular compartments and the plasma membrane (PM). Approximately half of intracellular GLUT4 is located in the ‘GLUT4 specialized compartment’ (SC) or ‘GLUT4 storage vesicle’ (GSV), which is segregated from the endocytic recycling compartment (ERC) and the trans-Golgi network (TGN). Insulin-regulated exocytosis pathway of GLUT4 is divided into (1) formation of SC/GSV, characterized by the presence of VAMP2; (2) movement of GLUT4 vesicles from the cell interior to the PM; (3) tethering of vesicles to the PM; and (4) fusion with the PM. Each step of the GLUT4 traffic may be regulated by insulin-derived signals.

10% is present at the cell surface (Sato *et al.* 1993, Li *et al.* 2001a). The steady-state distribution of GLUT4 at the basal state is maintained by fast endocytosis and slow exocytosis. Insulin shifts the steady-state distribution of GLUT4 favouring the PM by largely elevating the GLUT4 exocytic rate, a process known as GLUT4 translocation. GLUT4 resides in multiple intracellular compartments that include the trans-Golgi network (TGN) and the endocytic recycling compartment (ERC). However, approximately half of the GLUT4 molecules are located in an intracellular compartment, termed the ‘GLUT4 specialized compartment’ (SC) or ‘GLUT4 storage vesicle’ (GSV), which is segregated from the ERC and the TGN (Livingstone *et al.* 1996, Ueyama *et al.* 1999, Zeigerer *et al.* 2002). Although the biochemical and structural nature of the SC/GSV still remains undetermined, it is known that insulin stimulates the exocytosis of GLUT4 from this compartment. The insulin-regulated exocytosis of GLUT4 can be divided into: (1) formation of SC/GSV from the ERC and/or TGN (Watson *et al.* 2004, Shi & Kandror 2005), (2) movement of vesicles from the cell interior to the PM along a microtubule network (Patki *et al.* 2001, Semiz *et al.* 2003), (3) tethering near/to the PM prior to fusion (Inoue *et al.* 2003, Ewart *et al.* 2005) and (4) soluble NSF attachment protein receptor (SNARE) complex-mediated docking and fusion with the PM (Foster & Klip 2000) (Fig. 1). However, despite the advances in our understanding of the molecular mechanism of GLUT4 traffic, the specific steps in GLUT4 exocytosis regulated by insulin remain contro-

versial. It is conceivable that G proteins may be key regulators responding to insulin signals, thereby lying at the crossroads of signalling and vesicle traffic.

Insulin signalling cascade directing GLUT4 traffic

Considerable effort has focused on identifying the insulin signalling cascade that leads to GLUT4 translocation. To date, such insulin-derived signals are largely within the insulin receptor-insulin receptor substrate-phosphatidylinositol 3-kinase (IR-IRS-PI3K) axis (Fig. 2). Insulin-binding to the IR causes conformational change in the receptor, stimulating its intrinsic tyrosine kinase activity (Ottensmeyer *et al.* 2000). IRS binds to the phosphorylated IR and becomes tyrosine phosphorylated by IR. Out of six isoforms, IRS-1 is essential for GLUT4 translocation in muscle cells (Thirone *et al.* 2006). Class I PI3K docks onto the phosphorylated IRS and is thereby activated. PI3K-derived phosphatidylinositol 3,4,5-trisphosphate (PIP₃) recruits and activates the Ser/Thr kinases Akt (Wang *et al.* 1999, Bae *et al.* 2003) and atypical protein kinase Cs (aPKCs) ζ and λ (Bandyopadhyay *et al.* 1999, Sajan *et al.* 2006). These kinases are also required for GLUT4 translocation. Recently, a substrate for Akt involved in GLUT4 translocation was identified, and termed AS160 (Kane *et al.* 2002). AS160 is a GTPase-activating protein (GAP) for Rab small G proteins (Miinea *et al.* 2005).

In addition, the activation of PI3K is also essential for the activation of Rac1, a Rho family small G protein also

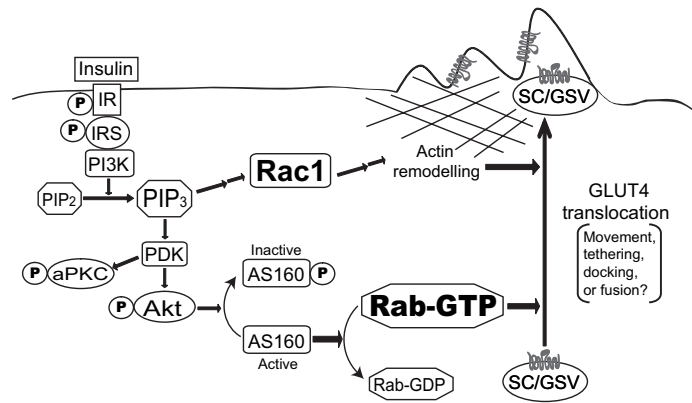


Figure 2 Insulin signalling cascade regulating glucose transporter 4 (GLUT4) traffic. Insulin-derived signals are largely within the insulin receptor-insulin receptor substrate-phosphatidylinositol 3-kinase (IR-IRS-PI3K) axis. This signalling cascade bifurcates downstream of PI3K into an arm leading to Akt/aPKC activation and another leading to Rac1 activation. PI3K-derived PIP₃ mediates the activation of Ser/Thr kinases Akt and atypical protein kinase Cs (aPKCs). The activated Akt phosphorylates and inactivates Rab-GAP AS160, and thereby the level of active (GTP-bound) Rabs is increased. The activation of PI3K is also essential for Rac1 activation, which is involved in actin remodelling.

involved in GLUT4 translocation (JeBailey *et al.* 2004, 2007) (Fig. 2). The activation of Rac1 is important for the remodelling of actin, which is a necessary component for insulin-induced GLUT4 translocation (Khayat *et al.* 2000, Patel *et al.* 2006). Insulin-induced actin remodelling is a process of dynamic changes in cortical filamentous actin documented in muscle and fat cells.

We have recently shown that the insulin signalling cascade bifurcates downstream of PI3K into an arm leading to the Akt/aPKC activation and another leading to Rac1 activation (Fig. 2). Specifically, neither dominant-negative mutants of Akt nor non-phosphorylatable AS160 prevents actin remodelling (Wang *et al.* 1998b, Thong *et al.* 2007), and conversely Rac1 knockdown does not alter Akt phosphorylation (JeBailey *et al.* 2007). However, the pathway downstream of Rac1 is still largely unknown. Likewise, the events downstream of AS160 are only beginning to be unravelled.

Small G proteins

Small G proteins function as GDP/GTP-regulated molecular switches that interconvert between an inactive GDP-bound form and an active GTP-bound form (Vetter & Wittinghofer 2001). In the active form, small G proteins interact with a large number of specific effector proteins to carry out their diverse physiological roles. Their GDP/GTP cycling is highly controlled by two types of regulatory proteins, as small G proteins possess low intrinsic GTP hydrolysis and GDP/GTP exchange activities. Guanine-nucleotide exchange factor (GEF) promotes the formation of the GTP-bound form (Schmidt & Hall 2002), whereas GAP accelerates the intrinsic GTPase activity to promote the GDP-bound form (Bernards & Settleman 2004). The

small G protein super-family is divided into four major branches on the basis of sequence and functional similarities: Ras, Rab, Rho and Arf families (Wennerberg *et al.* 2005). The Rab family primarily plays a role in vesicle traffic, while the Rho family is mainly involved in actin cytoskeleton organization. As stated above, both these processes are involved in GLUT4 translocation. Consistent with this tenet, GTP- γ -S, a non-hydrolysable GTP analogue, stimulates GLUT4 translocation (Kanai *et al.* 1993). Moreover, specific small G proteins of the Rab and Rho families engage in insulin-stimulated GLUT4 translocation. Here we describe the roles of Rab and Rho proteins in GLUT4 traffic.

Rab family in GLUT4 traffic

Rab proteins are localized to distinct intracellular compartments and regulate transport between organelles (Zerial & McBride 2001). In humans, the Rab family consists of almost 70 members. The large size of the Rab family in mammals reflects the complexity of transport events. Several mammalian Rabs are expressed in a tissue and cell-type specific manner, where they participate in specialized transport pathways. Rab proteins are involved in each step of the vesicular transport, which includes the formation and movement of vesicles, as well as tethering and fusion of vesicles with acceptor membranes. The activities of Rabs are highly regulated by GEFs and GAPs, as well as other small G protein family members. In addition, the localization and activity of Rab proteins is determined by their association/dissociation with diverse Rab-GDP dissociation inhibitors (Rab-GDIs) (Seabra & Wasmeier 2004). Rab-GDIs extract GDP-Rab from the membrane and form stable cytosolic complexes. Rabs are delivered

to their target membranes by Rab-GDIs, where they are activated by specific GEFs.

There have been numerous attempts to identify the specific Rab proteins required for GLUT4 translocation. While there do not appear to be muscle or adipose cell-specific Rab proteins, Rabs 2, 4, 8, 10, 11 and 14 were detected in isolated GLUT4-containing intracellular membranes from adipocytes and/or muscle cells (Cormont *et al.* 1993, Larance *et al.* 2005, Miinea *et al.* 2005). Of these, Rabs 4 and 11 were shown to be involved in GLUT4 traffic through the use of dominant-negative mutants (Vollenweider *et al.* 1997, Uhlig *et al.* 2005). In addition, Rab5 was also shown to participate in GLUT4 endocytic traffic (Huang *et al.* 2001). With the identification of the Akt substrate protein, AS160 as a Rab-GAP (Kane *et al.* 2002), the possibility emerged that signalling and traffic may meet at the level of the target Rab protein(s). Through the use of non-phosphorylatable mutants and gene silencing, the participation of AS160 in GLUT4 traffic in adipose and muscle cells, and in muscle tissue is now indisputable (Kane *et al.* 2002, Eguez *et al.* 2005, Larance *et al.* 2005, Kramer *et al.* 2006a, Thong *et al.* 2007). In addition to the AS160 connection, Gapex-5 was identified as a GEF regulating the activity of a Rab involved in GLUT4 traffic (Lodhi *et al.* 2007). Next we summarize the knowledge to date on the participation of Rabs 4, 5 and 11, and the target Rabs of Gapex-5 and AS160 in insulin-stimulated GLUT4 translocation.

Rab4

Rab4 is localized to the early/recycling endosomes, where it contributes to cargo sorting and recycling (Zerial & McBride 2001). Rab4 is also present in GLUT4-containing vesicles of skeletal muscle (Sherman *et al.* 1996) and adipocytes (Cormont *et al.* 1993). A body of evidence supports the participation of Rab4 in GLUT4 traffic, as follows. Insulin caused a decrease in Rab4 from the vesicular fraction in adipocytes (Cormont *et al.* 1993) and muscle (Le Marchand-Brustel *et al.* 1995, Sherman *et al.* 1996). In adipocytes, insulin stimulated GTP-loading on Rab4 in a PI3K-dependent manner (Shibata *et al.* 1997). GLUT4 translocation was inhibited by microinjection of a synthetic peptide corresponding to the C-terminal domain of Rab4 (Shibata *et al.* 1996), and by expression of wild-type, constitutively-active and prenylation-deficient Rab4 proteins (Cormont *et al.* 1996, 2001, Vollenweider *et al.* 1997). Moreover, insulin stimulated the interaction between Rab4 and a motor protein kinesin, downstream of aPKC activation (Imamura *et al.* 2003). This interaction is presumably required for the movement of GLUT4 vesicles to the PM. Finally, Rab4 directly interacts with the *t*-SNARE syntaxin4 (Li *et al.*

2001b) that mediates the fusion of GLUT4 vesicle with the PM (Foster & Klip 2000, Yang *et al.* 2001, Kanda *et al.* 2005). Taken together, Rab4 appears to be involved in both the movement of vesicles along the microtubule network and the fusion of the vesicles with the PM.

Rab5

In the endocytic pathway, Rab5 is localized to the PM and early endosomes (Zerial & McBride 2001). There, it mediates clathrin-coated vesicle-mediated transport from the PM to the early endosomes as well as homotypic, early endosome fusion. Microinjection of anti-Rab5 antibody into 3T3-L1 adipocytes increased GLUT4 levels at the periphery of unstimulated cells, while it did not affect insulin-stimulated GLUT4 translocation (Huang *et al.* 2001). This was presumably due to the inhibition of endocytosis towards the recycling compartments. Based on this evidence, Rab5 is thought to play a role in the endocytic part of GLUT4 traffic. Interestingly, knockdown of Rab5 by siRNA inhibits insulin-stimulated Akt phosphorylation by impairing the interaction between IRS1 and the p85 α subunit of the PI3K (Su *et al.* 2006), suggesting that Rab5 is also required for the fidelity of insulin signalling.

Rab11

Rab11 localizes to the ERC, TGN and PM, and controls recycling through the ERC and transport from the TGN to the PM (Zerial & McBride 2001). Rab11 is also present in GLUT4-containing vesicles in adipocytes (Larance *et al.* 2005) and cardiac muscle cells (Kessler *et al.* 2000) where insulin also stimulates glucose uptake. In rat cardiac muscle cells, Rab11 redistributed from the microsomal fraction to the PM fraction in response to insulin (Kessler *et al.* 2000). Moreover, expression of wild-type Rab11 decreased surface GLUT4 levels in the basal state, but did not affect its level after insulin stimulation (Uhlig *et al.* 2005). In contrast, dominant-negative Rab11 inhibited insulin-stimulated GLUT4 translocation, but did not affect basal GLUT4 levels. In adipocytes, expression of Rab11-binding protein, which interferes with Rab11 function, inhibited insulin-stimulated GLUT4 translocation and increased the amount of GLUT4 in the ERC (Zeigerer *et al.* 2002). Taken together, Rab11 is thought to be required for the transport of GLUT4 from the ERC to the SC/GSV.

Rab-GEF: Gapex-5

Recently, Gapex-5 was identified as a ligand of CIP4, a Cdc42 and TC10-interacting protein (Lodhi *et al.*

2007). TC10 contributes to GLUT4 traffic in 3T3-L1 adipocytes (Chiang *et al.* 2001). Over-expression of Gapex-5 diminished insulin-stimulated GLUT4 translocation. Gapex-5 functions as a GEF for Rab31, a member of the Rab5 subfamily. Over-expression of wild-type and constitutively-active Rab31 also inhibited insulin-stimulated GLUT4 translocation, whereas dominant-negative Rab31 on its own sufficed to raise surface GLUT4 suggesting that the traffic step controlled by this Rab may be an idle cycling between endosomal fractions, effectively retaining GLUT4 in intracellular compartments. Insulin leads to the recruitment of CIP4/Gapex-5 complex to the PM probably via TC10 activation, and decreases the GTP-bound form of Rab31 (Lodhi *et al.* 2007). Hence, Gapex-5 may regulate intracellular GLUT4 traffic by controlling Rab31 activity. Whether Gapex-5 or Rab31 responds to input from the PI3K pathway remains unknown.

Rab-GAP: AS160

AS160 was identified in a screen of Akt substrates (Kane *et al.* 2002). Structural features of AS160 are two phosphotyrosine binding (PTB) domains, six Akt-phosphorylation motifs and a TBC (Tre-2/Bub2/Cdc16) domain (Sano *et al.* 2003) with predicted Rab-GAP activity. In the basal state, AS160 localizes to the GLUT4 vesicles in its un-phosphorylated form. Insulin causes phosphorylation of AS160 at the Akt phosphorylation sites and its dissociation from GLUT4 vesicles to the cytosol (Larance *et al.* 2005). An AS160 mutant in which four of these phosphorylation sites are mutated to alanine (4P-AS160), inhibits insulin-stimulated GLUT4 translocation in adipocytes (Sano *et al.* 2003), muscle cells (Thong *et al.* 2007) and skeletal muscles (Kramer *et al.* 2006a), demonstrating that the phosphorylation of AS160 is essential for GLUT4 translocation. The TBC domain is conserved in all Rab-GAPs identified so far, except for Rab3A-GAP (Itoh *et al.* 2006). The TBC domain of AS160 displays Rab-GAP activity towards Rabs 2A, 8A, 10 and 14 *in vitro* (Miinea *et al.* 2005). A mutant of AS160 with lysine replacement at Arg973 (a residue deemed necessary for GAP activity) does not show *in vitro* GAP activity. Importantly, inhibition of GLUT4 translocation by 4P-AS160 is restored by introducing the mutation Arg973Lys into 4P-AS160, indicating that GAP activity is essential for the role of AS160 in GLUT4 translocation (Sano *et al.* 2003). The currently held model proposes that insulin-dependent phosphorylation of AS160 inhibits its GAP activity and leads to its dissociation from the GLUT4 vesicles, thereby increasing the active GTP-bound form of Rabs on GLUT4 vesicles and allowing or affecting GLUT4 translocation.

At present, there is controversy over the precise step(s) of GLUT4 traffic regulated by AS160. Using fluorescence quenching of transferrin-containing compartments, Zeigerer *et al.* (2004) showed that 4P-AS160 inhibits the insulin-induced shift of GLUT4 from the SC/GSV to the ERC and the PM. The authors further concluded that this protein participates in the dynamic intracellular retention of GLUT4, given that siRNA-mediated knockdown of AS160 expression elevated basal PM GLUT4 levels and glucose uptake, and caused a shift in the intracellular distribution of GLUT4 from the SC/GSV to ERC (Eguez *et al.* 2005). However, scrutiny of events closer to the PM reveals other functions of AS160. Total internal reflection fluorescence microscopy (TIRFM) has been applied to determine the dynamic behaviour of GLUT4 vesicles within ~250 nm of the membrane of 3T3-L1 adipocytes. Accordingly, Zeigerer *et al.* (2004) concluded that AS160 regulates GLUT4 translocation at a step preceding tethering/fusion of GLUT4 vesicles with the PM. When more amply analysed, TIRFM can also provide information on the velocity, immobilization (through tethering or docking) and fusion of the vesicles with the membrane (Lizunov *et al.* 2005, Gonzalez & McGraw 2006, Bai *et al.* 2007, Huang *et al.* 2007). By this approach, Bai *et al.* (2007) performed a detailed quantitative kinetic analysis and concluded that insulin causes immobilization of GLUT4 near/at the membrane and preferentially speeds up the time between such docking and actual fusion. In this study, 4P-AS160 interfered with the insulin-induced immobilization step. The authors concluded that 4P-AS160 had interfered with docking events. Conversely, Huang *et al.* (2007) interpreted the insulin-induced immobilization as a result of interaction with clathrin after fusion with the membrane. Therefore, the present evidence suggests that AS160 may regulate both the movement and the tethering steps, but not the fusion step of GLUT4 translocation.

How could phosphorylation of AS160 perform such diverse functions? One may speculate that this could arise from inhibition of its GAP activity towards distinct Rab proteins. Rabs 2, 4, 8A, 10, 11 and 14 are found on purified GLUT4 compartments (Cormont *et al.* 1993, Larance *et al.* 2005, Miinea *et al.* 2005), and Rabs 2, 8A, 10 and 14 are *in vitro* targets of the AS160 TBC domain (Miinea *et al.* 2005). Accordingly, we speculated that one or more of these Rabs might act downstream of AS160 phosphorylation to promote GLUT4 translocation (Ishikura *et al.* 2007). We tested this concept through a gain-of-function paradigm, in L6 muscle cells stably expressing *myc*-tagged GLUT4 (GLUT4*myc*). This cell line allowed us to quantify fully-inserted GLUT4 molecules at the PM in non-permeabilized cells by immunodetection of the *myc* tag.

The suitability of L6 GLUT4*myc* myoblasts as a model for studying GLUT4 traffic is extensively reviewed in Rudich & Klip (2003). As mentioned above, 4P-AS160 reduced the level of cell-surface GLUT4 in the basal and insulin-stimulated states. We observed that co-expression of Rab 8A (wild-type or constitutively-active) or of wild-type Rab 14 with 4P-AS160 prevented the reduction in cell surface GLUT4 in the insulin-stimulated state caused by 4P-AS160 alone (Fig. 3). In contrast, Rab10 was unable to rescue the inhibitory action of 4P-AS160 (Ishikura *et al.* 2007). Rab2 was not tested in this experimental paradigm as it normally mediates events at the level of the ER, and GLUT4 cycling occurs at the level of ERC/TGN and the PM. The results illustrated suggest that Rabs 8A and 14 may be physiological substrates of AS160. We next discuss how Rab8A and Rab14 might contribute to GLUT4 traffic, based on their known function in other cell systems.

Rab8A. Rab8A localizes to both the TGN and the cell periphery, and regulates the polarized transport of membrane proteins from the TGN to the PM (Peranen *et al.* 1996, Ang *et al.* 2003). Rab8A shows the highest homology to Sec4p, a yeast Rab small GTPase required

to tether vesicles mobilized from the TGN to the PM by interacting with Sec10p and Sec15p, components of the exocyst complex (Sztul & Lupashin 2006). Indeed, the exocyst complex has been proposed to be required to tether GLUT4 vesicles (Inoue *et al.* 2003, Ewart *et al.* 2005). Hence, we speculate that AS160 may regulate the tethering step of insulin-stimulated GLUT4 translocation through Rab8A activity.

Rab14. Rab14 resides in the TGN and endosomes [phagosome, early endosomes (EE) and EE associated vesicles], and regulates traffic among these compartments (Junutula *et al.* 2004, Proikas-Cezanne *et al.* 2006). Hence, we speculate that Rab14 may be involved in the intra-endosomal traffic of GLUT4 vesicle between the ERC and SC/GSV and regulate the formation of SC/GSV and/or the movement of the GLUT4 vesicles to the PM.

Rab 10. Recently, Sano *et al.* (2007) demonstrated that Rab10 is required for insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes. Over-expression of constitutively-active Rab10 increased surface GLUT4 at the basal state. In contrast, Rab10 knockdown via siRNA reduced insulin-stimulated GLUT4 transloca-

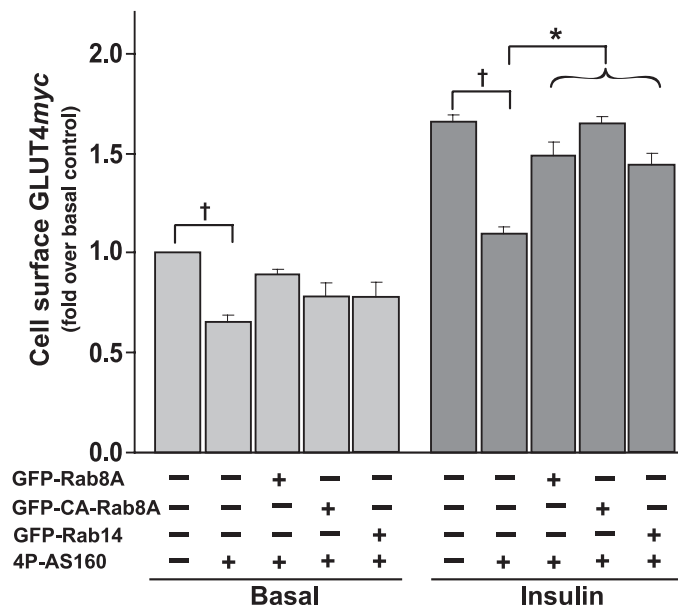


Figure 3 Rabs 8A and 14 rescue the inhibition of glucose transporter 4 (GLUT4) translocation caused by 4P-AS160. L6GLUT4*myc* myoblasts were transiently transfected with the indicated constructs, then serum-deprived and left unstimulated (basal) or stimulated with 100 nM insulin for 20 min. Cell surface GLUT4*myc* content was measured by detection of anti-*myc* antibody immunoreactivity in non-permeabilized cells. Cells expressing 4P-AS160 were identified by FLAG-antibody following permeabilization. Fluorescent images were obtained with Zeiss LSM 510 laser scanning confocal microscopy (Carl Zeiss, Oberkochen, Germany). Pixel intensity of cell surface GLUT4*myc* in single cells was quantified by IMAGE J software. Raw data were converted to fold-change above basal levels relative to surface GLUT4*myc* signal in untransfected, vicinal cells. Data are mean \pm SEM and expressed relative to the values in basal control cells. † $P < 0.05$ vs. control (within treatment). * $P < 0.05$ vs. 4P-AS160 alone (within treatment). Results adapted from Ishikura *et al.* (2007).

tion. Finally, the basal increase in surface GLUT4 resulting from AS160 knockdown was partially blocked by knocking down Rab10. These results suggest that Rab10 is a target of AS160 in adipocytes. The target Rabs of AS160 involved in insulin-stimulated GLUT4 translocation may differ between muscle and adipose cells. Further studies are needed to fully elucidate the role(s) of AS160 and its target Rabs in GLUT4 traffic.

AS160-binding proteins

Several AS160-binding proteins have been identified, which may regulate its activity. These proteins are the insulin-regulated aminopeptidase (IRAP), calmodulin and certain 14-3-3 isoforms.

IRAP. The second PTB domain of AS160 directly interacts with carboxyl-terminal of IRAP (Larance *et al.* 2005, Peck *et al.* 2006). IRAP is a major component of SC/GSV that redistributes to the PM in response to insulin along with GLUT4 (Keller 2003). Yeh *et al.* (2007) suggested that IRAP expression is required for efficient GLUT4 translocation to the membrane in response to insulin. The interaction between AS160 and IRAP is not regulated by insulin or by the phosphorylation of AS160. One might speculate that AS160 is localized to GLUT4 vesicles through its interaction with IRAP, where it gains access to the cohort of vesicular Rabs.

Calmodulin. Calmodulin binds to a small domain preceding the TBC domain (Kane & Lienhard 2005). Calmodulin binding to AS160 is Ca²⁺-dependent, but not insulin-dependent. As an AS160 mutant that cannot bind to calmodulin (Leu842Gly and Trp843Gly double mutant) does not interfere with GLUT4 translocation by insulin, calmodulin binding is probably not required

for the function of AS160 in this process. On the other hand, it is conceivable that calmodulin may participate in signalling by AS160 elicited by stimuli other than insulin. Indeed, it has been shown that diverse stimuli cause a gain in surface GLUT4, such as muscle contraction, exercise, platelet-derived growth factor and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside. Notably, all these stimuli also induced AS160 phosphorylation in mature muscle, L6 muscle cells and Chinese hamster ovary cells over-expressing human insulin receptor (CHO-IR) cells (Kramer *et al.* 2006a,b, Thong *et al.* 2007). Importantly, 4P-AS160 expression inhibited the increase in glucose uptake or GLUT4 translocation by these stimuli, suggesting that AS160 may be a convergence factor for the distinct stimuli. As an intracellular [Ca²⁺] increase is especially important for contraction-stimulated GLUT4 translocation, calmodulin binding to AS160 may participate in GLUT4 translocation in response to this important stimulus.

14-3-3. Ramm *et al.* (2006) found that several 14-3-3 isoforms bind to a region containing the phosphorylated Thr642 in AS160 (one of the Akt phosphorylation sites). Insulin increased the interaction between AS160 and 14-3-3 in the 3T3-L1 adipocytes (Ramm *et al.* 2006) and in human skeletal muscle (Howlett *et al.* 2007). Such binding did not affect the interaction of AS160 with IRAP. It is speculated that insulin-dependent binding of 14-3-3 to AS160 may affect its GAP activity rather than the dissociation of AS160 from GLUT4 vesicles, an idea amenable to experimental scrutiny. It is not known at present whether the interaction of AS160 with IRAP, calmodulin or 14-3-3 alters its GAP activity, its susceptibility to insulin regulation or its accessibility to target Rabs.

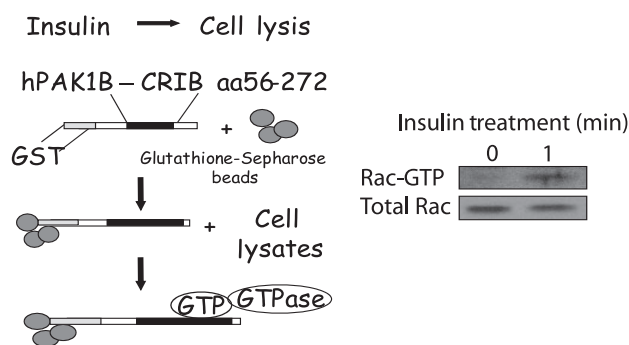


Figure 4 Insulin-induced Rac1 activation in L6 muscle cells. L6 myoblasts were serum deprived and exposed to 100 nM insulin for 1 min. GTP-bound Rac1 was precipitated using glutathione-S-transferase fusion protein of the p-21 kinase CRIB (Cdc42/Rac interactive binding) domain conjugated to glutathione-sepharose beads. The insulin-activated, GTP-bound Rac1 and the total Rac1 from cell lysates were detected by immunoblotting. Insulin-induced Rac1 activation was readily detected after 1 min of stimulation and was sustained for up to 10 min (data not shown).

Actin remodelling in insulin-induced GLUT4 translocation

Insulin-stimulated GLUT4 translocation requires a dynamic modification of actin filaments, termed actin remodelling. This concept has been demonstrated using pharmacological actin depolymerizing (latrunculin B, cytochalasin D) and actin stabilizing (jasplakinolide, swinholide A) agents, which in turn inhibited insulin-stimulated GLUT4 translocation (Tsakiridis *et al.* 1994, Khayat *et al.* 2000, Tong *et al.* 2001). In response to insulin, actin fibres remodel into an actin mesh beneath the PM in muscle cells, creating membrane ruffles or lamellipodia. This remodelling takes place within 3 min of insulin stimulation (Khayat *et al.* 2000). Actin remodelling also takes place in 3T3-L1 adipocytes where intense changes in polymerization are noted at both the cell cortex and perinuclear regions (Wang *et al.* 1998a, Kanzaki & Pessin 2001). Two scenarios have been suggested for the function of the actin mesh in the insulin-dependent GLUT4 traffic. (1) Remodelled actin may serve as a scaffold that collects and orients selective signalling molecules for the proper signal transduction. This model is supported by the co-localization of signalling molecules such as IRS, PI3K and Akt with the remodelled actin after insulin treatment in L6 cells (Khayat *et al.* 2000, Tong *et al.* 2001). (2) Remodelled actin may serve as tracks for motor proteins that pull the GLUT4 vesicles towards the PM (Emoto *et al.* 2001).

The induction of insulin resistance in muscle cell reduced insulin-induced actin remodelling, further emphasizing its physiological and cellular importance (Tong *et al.* 2001, JeBailey *et al.* 2004). Rho family small G proteins play a pivotal role in reorganization of the actin cytoskeleton, and may be targets of insulin resistance-causing agents.

Rho family: Rac1, Cdc42, Rho A, TC10 in insulin action

In mammals, the Rho family of G proteins encompasses 22 different proteins, and the three best characterized are Rac1, Cdc42 and RhoA. Rac1 induces the formation of lamellipodia or membrane ruffling (Etienne-Manneville & Hall 2002). Cdc42 induces the formation of filopodia, while RhoA induces stress fibre formation (Etienne-Manneville & Hall 2002). The Rho GTPase, TC10, has cellular effects that most closely relate to those of Cdc42 (Neudauer *et al.* 1998). As mentioned earlier, insulin-stimulated GLUT4 translocation requires dynamic changes in the actin cytoskeleton structures. Interestingly, this behaviour appears to be elicited by different Rho small G proteins in muscle and adipose cells: Rac1 in muscle cells and TC10 in adipose cells.

TC10. TC10 transfected into 3T3-L1 adipocytes becomes activated (GTP loaded) in response to insulin, downstream of the Cap/Cbl/C3G cascade rather than PI3K signalling (Chiang *et al.* 2001). In that cascade, the insulin receptor in caveolae couples to and phosphorylates Cbl via Cap, thereby recruiting CrkII and C3G to the caveolae. C3G is the proposed GEF for TC10. TC10 was shown to be required for actin dynamics, insulin-stimulated GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes, through the use of dominant-negative mutants (Chiang *et al.* 2001, Kanzaki *et al.* 2002). On the other hand, such mutants were without effect on actin remodelling and GLUT4 translocation in L6 myoblasts, albeit causing alterations in the basal actin morphology (JeBailey *et al.* 2004). The endogenous level of TC10 protein is rather low in muscle cells (JeBailey *et al.* 2004). TC10 transfected into L6 myoblasts became GTP loaded within minutes of insulin stimulation, in spite of the absence of Cap at that stage of the culture. Recombinant TC10 elicited comet tails on GLUT4 vesicles in a semi *in vitro* system (Kanzaki *et al.* 2001). TC10 has high sequence homology to Cdc42 and Rac1 and can activate proteins that are also targets of these G proteins (Kanzaki 2006). However, the search for downstream effectors of TC10 is still ongoing, as well as its relevance to GLUT4 traffic in muscle tissue.

Cdc42. The involvement of Cdc42 GTPase in insulin-stimulated gain of GLUT4 at the PM is not firmly established, as there are conflicting results in terms of its activation in response to insulin and its involvement in actin remodelling and GLUT4 translocation. Expression of constitutively-active, wild-type and dominant-negative Cdc42 in 3T3-L1 adipocytes had no effect on the cortical actin structures and insulin-stimulated GLUT4 translocation (Chiang *et al.* 2001, Kanzaki *et al.* 2002). In contrast, Cdc42 knockdown via siRNA or microinjection of anti-Cdc42 antibody significantly decreased insulin-stimulated GLUT4 translocation (Usui *et al.* 2003). This was demonstrated to take place downstream of a $G_{\alpha q/11}$ (that can be activated by bradykinin leading to an increase glucose uptake and actin remodelling; Kishi *et al.* 1998) and to be PI3K- and PKC λ -dependent (Usui *et al.* 2003).

RhoA. The involvement of RhoA in insulin-induced GLUT4 translocation is still poorly mapped. In adipocytes, RhoA is GTP-loaded and a significant portion of this GTPase is translocated to the PM in response to insulin (Karnam *et al.* 1997). Inhibition of PI3K by wortmannin or LY294002 inhibited the translocation of RhoA, but not its activation (Karnam *et al.* 1997). The use of C3 transferase, a selective inhibitor of RhoA, decreased insulin-induced glucose uptake in 3T3-L1

cells (Karnam *et al.* 1997). The transfection of constitutively-active RhoA increased the co-transfected HA-GLUT4 translocation to the PM in response to insulin, while dominant-negative RhoA significantly decreased it (Standaert *et al.* 1998). On the other hand, Chiang *et al.* (2001) could not see any effect of transfected wild-type, constitutively-active or dominant-negative RhoA on insulin-stimulated GLUT4 translocation and glucose uptake in the same cells. In accordance with this, transfection of dominant-negative RhoA into L6 cells was without effect on insulin-induced actin remodelling (Khayat *et al.* 2000).

Rac1. The participation of Rac1 in GLUT4 translocation in adipocytes still awaits experimental scrutiny. An earlier study had suggested that Rac1 is not involved in the insulin-induced stimulation of glucose uptake in 3T3-L1 adipocytes, through transfection of dominant-negative and constitutively-active versions of the protein (Marcusohn *et al.* 1995). Dominant-negative Rac1 failed to prevent insulin-dependent glucose uptake and constitutively-active Rac1 failed to rescue the inhibition caused by wortmannin (Marcusohn *et al.* 1995). However, there was no confirmation that the mutants, transfected in the fibroblast stage of the culture, persisted in the adipocyte stage where insulin-stimulated glucose uptake was studied. Moreover, it is not expected that constitutively-active Rac1 would restore the entire signalling to GLUT4, as signalling downstream of PI3K bifurcates into the Akt and the Rac/actin pathways.

In sharp contrast to the case with 3T3-L1-adipocytes, there is an increasing body of evidence supporting the activation of endogenous Rac1, in response to insulin in other cells (Ridley *et al.* 1992). This event, observed in the L6 muscle cells, is seen as early as within 1 min of insulin stimulation (Fig. 4). Such early activation is compatible with the rapid actin remodelling observed within 90 s of insulin action, and GLUT4 translocation that is discernible within 3–5 min of addition of the hormone. There is now good evidence that Rac1 activation is indeed necessary for the insulin-dependent gain in GLUT4 at the cell surface of L6 myoblasts and myotubes. Expression of a dominant-negative mutant of Rac1 in L6 myoblasts did not affect the basal amount of GLUT4 at the PM, while fully abolishing the increase in GLUT4 seen with insulin treatment (Khayat *et al.* 2000, JeBailey *et al.* 2004). In addition, dominant-negative Rac1 prevented cortical actin remodelling in response to insulin, without affecting the overall appearance of actin stress fibres in the basal or insulin-stimulated states (Khayat *et al.* 2000, JeBailey *et al.* 2004). Further supporting the participation of Rac1 in actin remodelling and GLUT4 traffic in L6 myotubes are results where expression of the protein was knocked down by

70% through siRNA transfection (JeBailey *et al.* 2007). These results emphasize the importance of functioning Rac1 in transducing the insulin stimulus into actin dynamics and subsequent GLUT4 translocation in muscle cells.

Signals leading to Rac activation and bifurcation of the PI3K cascade

Insulin-induced actin remodelling in L6 muscle cells is prevented by expression of the dominant-negative mutant of PI3K, high expression of a PIP₃ ligand (GFP-linked to the GRP-PH domain), and by pre-treatment with wortmannin or LY294002 (Tsakiridis *et al.* 1997, Patel *et al.* 2003, Rudich & Klip 2003). Similarly, wortmannin prevented insulin-dependent Rac1 activation (JeBailey *et al.* 2004). Interestingly, a dominant-negative mutant of Akt decreased insulin-induced GLUT4 gain at the PM, but had no effect on actin remodelling (Wang *et al.* 1999). This evidence suggested a bifurcation of the signalling pathway downstream of PI3K and PIP₃, so that Akt and Rac1 activation occur in parallel. To further this point, siRNA-mediated knockdown of Rac1 did not prevent insulin-induced Akt phosphorylation (JeBailey *et al.* 2007). Moreover, the two signalling arms seem to have different susceptibility to two agents that induce insulin resistance (JeBailey *et al.* 2007). Ceramide is a fatty acid derivative that accumulates in muscles of insulin-resistant humans (Adams *et al.* 2004), and exogenous administration of short-chain ceramide (C2) to muscle and adipose cells elicits insulin resistance. In the presence of glucose, glucose oxidase produces low levels of peroxide that trigger insulin resistance (Bloch-Damti & Bashan 2005, Houstis *et al.* 2006). Both glucose oxidase and C2 prevented Rac1 and Akt activation, without affecting upstream steps in insulin signalling (specifically IRS-1 phosphorylation and PI3K activation). However, consistently, Rac1 and actin remodelling were more susceptible than Akt phosphorylation to the inhibitory action of C2 and glucose oxidase. Whether these effects arise from changes in local availability of PIP₃ or from distinct hits on proteins governing Rac and Akt, respectively, is the subject of future investigation.

Downstream signalling by Rho GTPases

What mediates the effects of Rho GTPases on actin dynamics? Downstream of the Rho GTPases lie the Wiskott-Aldrich syndrome protein (WASP) and WASP family Verproline-homologous (WAVE) family, p-21 activated kinases (PAKs) and Rho kinases (ROCK). These proteins transduce the signal from the Rho GTPases to the remodelling of actin. Activation of

WASP/WAVE by Rho GTPases leads to activation of the Arp2/3 complex, which induces branching of existing actin filaments (Millard *et al.* 2004). Interestingly, dominant-negative N-WASP attenuated insulin-stimulated actin polymerization and decreased GLUT4 translocation in 3T3-L1 adipocytes (Kanzaki & Pessin 2001, Jiang *et al.* 2002). On the other hand, the Ser/Thr kinase PAK is also activated by Rac1, Cdc42 and TC10 (Zhao & Manser 2005), leading to activation of LIM kinase (LIMK), which can then inactivate cofilin, an actin-severing protein (Edwards *et al.* 1999). RhoA also activates LIMK via the activation of ROCK (Maekawa *et al.* 1999, Sumi *et al.* 2001). Interestingly, PAK and LIMK are activated in response to insulin (Tsakiridis *et al.* 1994, Yang *et al.* 1998). Future work should unravel the potential contribution of these proteins to GLUT4 traffic.

Conclusions and perspectives

Insulin-stimulated GLUT4 translocation is a complex process that becomes impaired in insulin resistance and Type 2 diabetes mellitus. Elucidating the molecular basis of GLUT4 traffic is important to understanding these metabolic disorders. Here we highlighted the roles of Rab and Rho small G proteins in insulin-regulated GLUT4 traffic. Despite recent scientific advances, the precise exocytic steps regulated by insulin remain undetermined. Identifying Rab proteins involved in each step of the GLUT4 traffic pathway, and revealing the regulatory mechanisms of the activities of such Rabs by GAP and GEF proteins is paramount. Similarly, it is imperative to delineate the signalling events that lie upstream and downstream of the Rho G proteins leading to insulin-dependent actin remodelling. Combined efforts to study both of these G protein families will possibly lead to the elucidation of the complete insulin-stimulated GLUT4 translocation pathway, and to identify how signal transduction directly modifies individual steps in vesicle traffic.

Conflicts of interest

There is no conflict of interest associated with this article.

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