In: Glucose Uptake
Editors: Carter C. Johnson and Davis B. Williams
ISBN: 978-1-62618-670-5
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Chapter 9

The Ras Superfamily of Small GTP-Binding Proteins in Glucose Transporter Type 4-Mediated Glucose Uptake in Insulin-Responsive Tissues

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Abstract

Insulin stimulates the uptake of glucose in several target tissues, including skeletal muscle and adipose tissue, thereby contributing to the decline in the blood glucose level. Insulin signaling is mediated by a highly integrated network composed of a variety of signal transducing proteins. Induction of glucose uptake in skeletal muscle and adipose tissue primarily depends on the redistribution of the glucose transporter type 4 (GLUT4) from intracellular storage sites to the plasma membrane. This process consists of several discernible steps, such as vesicle transport, docking, and fusion, each of which is regulated rigorously by the signal transduction machinery. Despite intensive research for decades, mechanisms underlying the regulation of GLUT4-mediated glucose uptake remain largely elusive. The Ras superfamily of small GTP-binding proteins serves as a molecular switch in many facets of signal transduction processes in various types of mammalian cells. Emerging evidence supports the notion that insulin also employs small GTP-binding proteins in the intracellular signaling network to exert its actions in skeletal muscle and adipose cells. Two types of proteins, guanine nucleotide exchange proteins (GEFs) and GTPase-activating proteins (GAPs), regulate on/off switching of small GTP-binding proteins, respectively. In fact, diverse GEFs and GAPs have been characterized as key components in insulin signaling.

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Regulation of the Ras Superfamily of Small GTP-Binding Proteins

The Ras superfamily is comprised of over 150 signal transducing small GTP-binding proteins, which are classified into five subfamilies, i.e. Ras, Rho, Rab, Arf, and Ran families (Takai et al., 2001; Wennerberg et al., 2005). The molecular switch function of Ras superfamily small GTP-binding proteins depends on their bound guanine nucleotide species (Figure 1).

Virtually all small GTP-binding proteins exist in complex with GDP or GTP, and these two conformations interact with a different set of proteins. If the affinities of the protein for GDP and GTP are similar (and this is the case for many small GTP-binding proteins), the protein preferentially binds to GTP because the intercellular concentration of GTP is higher than that of GDP. Small GTP-binding proteins themselves catalyze the hydrolysis of the bound GTP (and thus are also called “GTPases”), generating the GDP-bound conformation and orthophosphate.

In general, only the GTP-bound conformation interacts with downstream effectors or target proteins, which are responsible for conveying the signal further downstream.

Therefore, guanine nucleotide exchange from GDP to GTP is considered to be a “switch on” process, whereas the hydrolysis of GTP is considered to be a “switch off” process. These two processes are regulated respectively by diverse regulatory proteins.

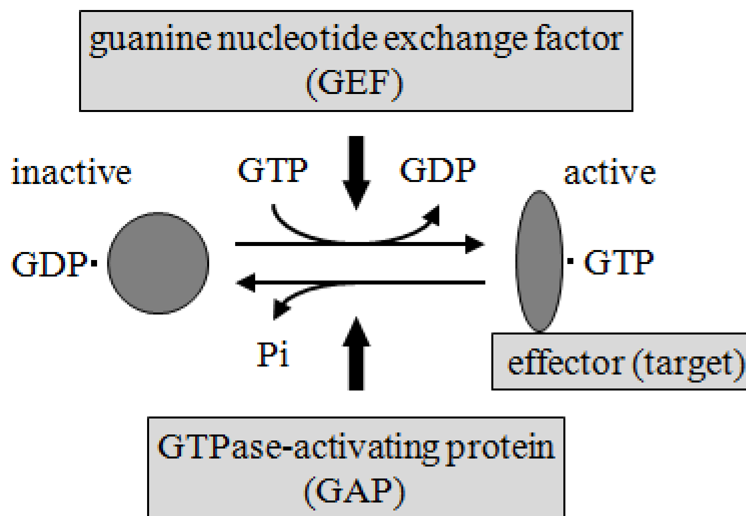

Figure 1. The GDP/GTP cycle of the small GTP-binding protein and its regulators. Small GTP-binding proteins exist in either the GDP-bound inactive or GTP-bound active conformation. GEFs stimulate the release of non-covalently bound GDP from the protein, leading to the accumulation of the GTP-bound conformation. The GTP-bound conformation specifically interacts with and activates downstream effector (target) proteins. GAPs enhance the GTPase activity of the GTP-binding protein. Thus, GEFs and GAPs act as positive and negative regulators for signal transduction, respectively. GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor.

The protein that enhances guanine nucleotide exchange from GDP to GTP is termed "guanine nucleotide exchange factor (GEF)", whereas the protein that enhances GTP hydrolysis is termed "GTPase-activating protein (GAP)".In many signal transduction pathways, the upstream signal is transmitted to a GTP-binding protein through a GEF, but in some cases, a GAP acts as an upstream regulator.

Various mutations naturally occur in genes that encode small GTP-binding proteins, causing cellular dysfunction. The most famous example of this is a GTPase-deficient mutant of Ras found in a variety of cancer cells.

In addition to such pathologically characterized mutants, many types of mutants have been generated by recombinant DNA techniques, being employed for the analysis of signal transduction mechanisms. These mutants of small GTP-binding proteins can be classified into two categories; one shows GTPase deficiency and another shows high affinity to GEFs. GTPase-deficient mutants exist in a GTP-bound conformation, continuously conveying the signal to downstream targets. Thus, this type of mutants is "constitutively active" in terms of signal transduction.

However, it should be noted that this is not always the case because, in some cases, both GTP binding and hydrolysis (i.e. continual GDP/GTP cycling) is required for exerting the signaling function. Mutants with high affinity to GEFs usually act in a "dominant-negative" manner by sequestering their GEFs.

GLUT4 in Insulin-Dependent Glucose Uptake Signaling

The glucose transporter type 4 (GLUT4) mediates insulin-dependent glucose uptake in skeletal muscle and adipose tissue (Huang and Czech, 2007; Saltiel and Kahn, 2001). GLUT4 is a 12-transmembrane protein that catalyzes glucose transport across the cell membrane by an ATP-independent facilitative diffusion mechanism (Huang and Czech, 2007). A pivotal role for GLUT4 in whole-body glucose homeostasis is strongly suggested by various genetically engineered mouse models (Huang and Czech, 2007).

For instance, mice with conditional deletion of GLUT4 in skeletal muscle or adipose tissue display impaired insulin responsiveness and diabetic phenotypes (Abel et al., 2001; Zisman et al., 2000). Thus, it is important to understand the signaling mechanisms whereby insulin stimulates glucose uptake through the modulation of GLUT4 activity.

GLUT4 is distributed in various membrane structures and dynamically recycles between intracellular stores and the plasma membrane (Figure 2). In unstimulated cells, GLUT4 is sequestered away from the endosomal recycling compartment (ERC) into a specialized intracellular compartment called GLUT4 storage vesicles (GSV) due to slow exocytosis (Karylowski et al., 2004).

Insulin accelerates exocytosis of GSV to the plasma membrane, leading to a net accumulation of GLUT4 on the cell surface. Multiple steps of exocytosis, including trafficking, tethering, docking, and fusion, are subjected to insulin regulation. Recently, it has been demonstrated that insulin also regulates GLUT4 vesicle formation, which is the earliest step of GLUT4 recycling (Li et al., 2012). Endocytosis of GLUT4, on the other hand, is

attenuated upon insulin stimulation, also contributing to plasma membrane distribution of GLUT4.

Following insulin stimulation, the activated insulin receptor triggers downstream signaling by phosphorylating a variety of target proteins. The adaptor protein IRS1 mediates the formation of the signaling complex.

A protein kinase cascade consisting of phosphoinositide 3-kinase (PI3K), PDK1, and Akt2 plays a pivotal role downstream of IRS1 for the induction of glucose uptake (Huang and Czech, 2007; Saltiel and Kahn, 2001; Figure 3). PI3K yields 3-phosphoinositides such as phosphatidylinositol 3,4,5-trisphosphate, which induce translocation of PDK1 and Akt2 to the plasma membrane.

Subsequently, PDK1 and the mammalian target of rapamycin complex 2 phosphorylate and activate Akt2. Akt2 has been implicated in several GLUT4 exocytic processes by phosphorylating specific targets (Franke, 2008; Vanhaesebroeck et al., 2012).

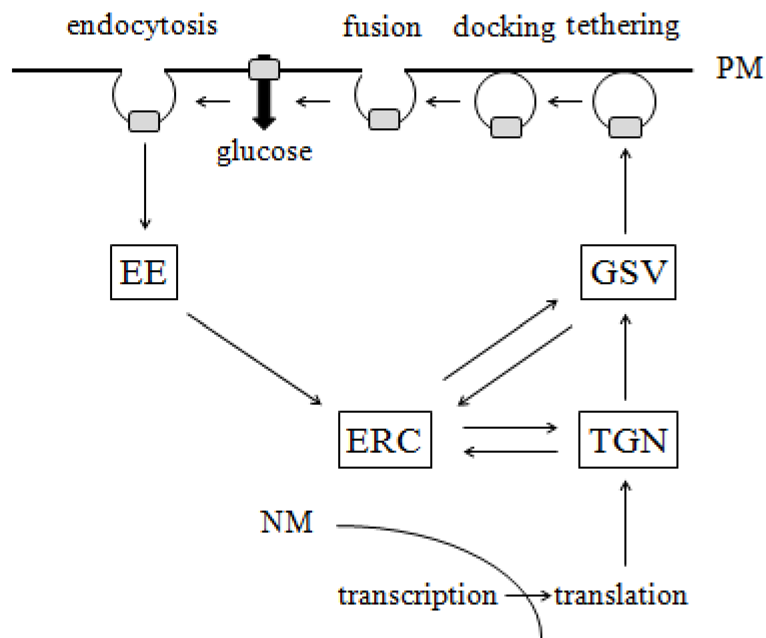

Figure 2. GLUT4 trafficking pathways. GLUT4 is sequestered in the specialized intracellular compartment GSV in unstimulated cells. Upon insulin stimulation, GLUT4 is redistributed, accumulating in the plasma membrane. Various endocytic and exocytic processes are subjected to insulin regulation. EE, early endosome; ERC, endosomal recycling compartment; GSV, GLUT4 storage vesicle; NM, nuclear membrane; PM, plasma membrane; TGN, trans-Golgi network. Gray squares represent GLUT4.

Rab Proteins

The Rab family of small GTP-binding proteins consists of over 60 members found in humans. Rab proteins have been implicated in the regulation of consecutive steps in intracellular membrane trafficking, including vesicle budding, delivery, tethering, and fusion with the target (acceptor) membrane. The specificity of membrane tethering and fusion is

particularly important for the proper flow of cargo within the cell (Zerial and McBride, 2001; Grosshans et al., 2006).

Like other small GTP-binding proteins, Rab proteins act as molecular switches, cycling between GDP-bound and GTP-bound states (Figure 4). In addition, Rab proteins undergo a cycle of membrane insertion and extraction, which is coupled with the above GDP/GTP cycle. The post-translational modification of C-terminal cysteines with geranylgeranyl moieties is prerequisite for membrane insertion.

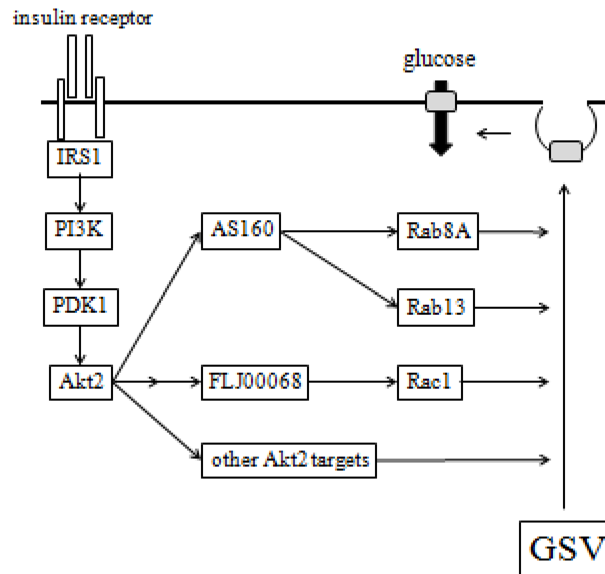

Figure 3. Signaling pathways downstream of the insulin receptor that regulate GLUT4 translocation to the plasma membrane in muscle cells. The ligand-bound insulin receptor stimulates a kinase cascade consisting of PI3K, PDK1, and Akt2. Akt2 is a master switch that regulates GLUT4 translocation in muscle cells. GSV, GLUT4 storage vesicle; PI3K, phosphoinositide 3-kinase.

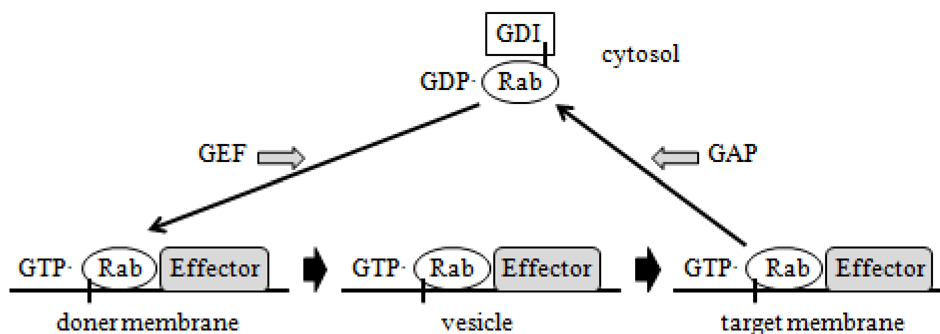

Figure 4. Membrane trafficking and the Rab protein cycle. Rab proteins cycle between membrane and cytosolic compartments. In membrane compartments, these proteins regulate vesicle transport through the action of various effectors. GDI recognizes the lipid moiety of Rab proteins, preserving these proteins in the cytosolic compartment. GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor.

Extraction from the membrane and preservation in the cytosol of lipid-modified Rab proteins require a protein termed GDP dissociation inhibitor (GDI), which masks the lipid moiety of the GDP-bound form of the Rab protein.

Once associated with membrane Rab proteins are subjected to the action of GEF, which catalyzes GTP binding to Rab proteins. The active, membrane-bound Rab protein interacts with specific effectors, exerting its functions. The switch is then turned off via hydrolysis of bound GTP, which is accelerated by specific GAPs, and the GDP-bound inactive Rab protein is recycled back to the cytosol (Zerial and McBride, 2001; Grosshans et al., 2006).

In adipocytes and skeletal muscle, Rab4 is localized not only to early endosomes, but also GLUT4-containing vesicles, suggesting a role for Rab4 in GLUT4 translocation (Cormont et al., 1996; Sherman et al., 1996; Vollenweider et al., 1997).

In fact, decrease in insulin-dependent GLUT4 translocation was observed upon inhibition of Rab4, and insulin stimulated GTP loading to Rab4 in PI3K-dependent manner (Cormont et al., 1996; Shibata et al., 1997). Activated Rab4 interacts with the kinesin motor KIF3, regulating GLUT4 vesicle transport along microtubules (Imamura et al., 2003). Therefore, Rab4 may participate in the subcellular redistribution of GLUT4 in insulin-stimulated conditions. However, molecular mechanisms whereby insulin regulates Rab4 remain obscure.

Rab5 regulates the early endocytic transport from the plasma membrane to early endosomes mediated by clathrin-coated vesicles (Zerial and McBride, 2001).

Inhibition of Rab5 increased the basal level of surface GLUT4 and reduced GLUT4 internalization after insulin removal (Huang et al., 2001). Thus, Rab5 may be involved in endocytic removal of cell surface GLUT4.

Another Rab family member Rab11 is required for GLUT4 trafficking from recycling endosomes to the specialized compartment and the insulin-induced translocation of GLUT4 to the plasma membrane (Uhlig et al., 2005; Zeigerer et al., 2002).

Rab31 is a member of the Rab5 subfamily, which is implicated in the regulation of membrane trafficking from the trans-Golgi network to endosomes. Dominant-negative Rab31 increased the basal level of GLUT4 accumulation in the plasma membrane, whereas overexpression of wild-type or constitutively active Rab31 blocked insulin-stimulated GLUT4 translocation (Lodhi et al., 2007).

Furthermore, knockdown of Rab31 significantly potentiated GLUT4 translocation stimulated by insulin. The formation of the GTP-bound form of Rab31 was inhibited by insulin. Taken together, insulin may permit GLUT4 vesicles to escape the futile intracellular cycle between endosomal fractions and to be translocated to the plasma membrane by inactivating Rab31 (Lodhi et al., 2007). A current model for the mechanism by which insulin inhibits Rab31 will be described below.

Rab10 is also present in GLUT4 vesicles, and is reported to have a pivotal role in insulin-stimulated translocation of GLUT4 to the adipocyte plasma membrane (Sano et al., 2007). GTPase-deficient Rab10 increased the cell surface level of GLUT4, whereas insulin-dependent GLUT4 translocation was attenuated when Rab10 was knocked down.

Importantly, Rab10 is a substrate of AS160, a Rab-GAP regulated through phosphorylation by Akt2, and the increase in the basal level of GLUT4 in the plasma membrane due to AS160 knockdown was restored by knocking down Rab10. Collectively, Rac10 is a insulin-responsive regulator of GLUT4 translocation, acting downstream of AS160 (Sano et al., 2007; see below). Rab8A, Rab10, and Rab13 belong to the same subfamily of

Rab GTP-binding proteins, and are substrates of AS160. Thus, it is likely that Rab8A and Rab13 are also implicated in the regulation of insulin-dependent GLUT4 trafficking.

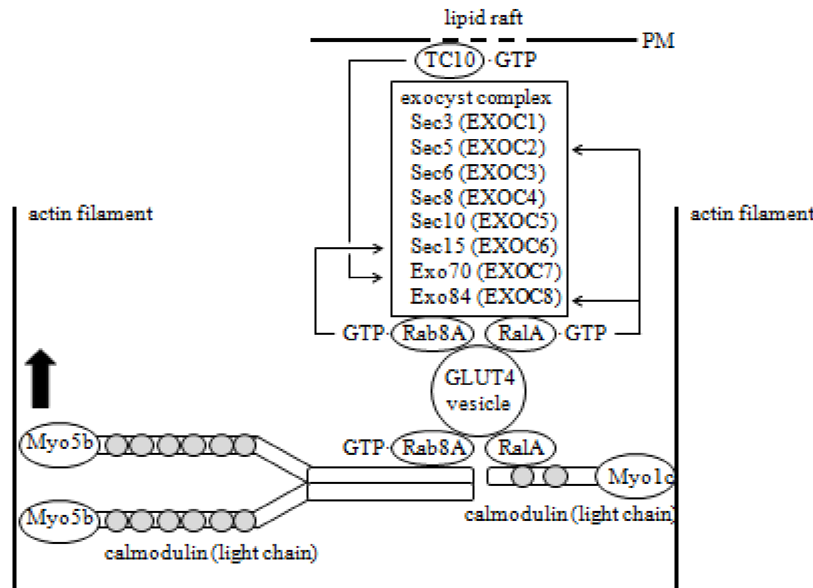

Figure 5. GULT4 vesicle transport driven by myosin motor proteins and exocyst complex-mediated tethering of the GLUT4 vesicle to the plasma membrane. Plasma membrane-localized TC10 anchors the exocyst complex beneath the plasma membrane. Rab8A and RalA contribute to the association of the GLUT4 vesicle to the exocyst complex. Rab8A and RalA also link the GLUT4 vesicle to Myo5b and Myo1c motors, respectively.

In fact, Rab8A and Rab13 are expressed in skeletal muscle cells, and become activated upon insulin stimulation (Sun et al., 2010). Furthermore, Rab8A and Rab13 can suppress the inhibitory effect of a dominant-negative AS160 mutant (Sun et al., 2010).

Taken together, Rab10 in adipocytes and Rab8A and Rab13 in muscle cells, respectively, are likely to be responsible for insulin-induced GLUT4 translocation downstream of AS160.

Rab8A can interact with the exocyst component Sec15 (Wu et al., 2005; Bryant et al., 2010), and thus it may contribute to GLUT4 vesicle tethering to the plasma membrane in skeletal muscle (Figure 5). However, it remains unclear whether Rab10 is also responsible for the interaction between the GLUT4 vesicle and the exocyst complex in adipocytes. Another effector for Rab8A and Rab10 is the motor protein Myo5b (Roland et al., 2007; Roland et al., 2011). In fact, engagement of Rab8A and Myo5b is important for insulin-induced GLUT4 translocation in muscle cells (Ishikura and Klip, 2008; Figure 5).

Rab GEFs

Although various Rab proteins have been implicated in GLUT4 vesicle trafficking, underlying regulatory mechanisms remain largely unknown. Given that many small GTP-binding proteins are activated in response to upstream signals through the action of their specific GEFs, Rab GEFs are possible candidates for direct activators. Indeed, a GEF for

Rab10 called Dennd4C is localized in GLUT4 vesicles, and its knockdown markedly diminished insulin-induced GLUT4 translocation (Sano et al., 2011). Thus, this GEF may be responsible for insulin-dependent activation of Rab10 in adipocytes. To my knowledge, GEFs that act on Rab8A and Rab13 in insulin-stimulated muscle cells remain unidentified. Another example of the involvement of a GEF in Rab regulation is insulin-dependent sequestration of the GEF Gapex-5 from its substrate Rab31 in adipocyte-specific TC10-mediated signaling (Lodhi et al., 2007; see below).

Rab GAPs

Through screening Akt substrates, a TBC domain-containing Rab-GAP termed AS160 (TBC1D4) was identified (Kane et al., 2002). A subfamily of Rab proteins consisting of Rab8A, Rab10, and Rab13 is known to be a target of AS160, being involved in insulin signaling. AS160 is associated with GLUT4 vesicles in unstimulated cells, and becomes dissociated following insulin stimulation (Larance et al., 2005). Five amino acid residues of AS160 are phosphorylated by Akt in insulin-stimulated adipocytes (Sano et al., 2003). Phosphorylation of AS160 is also detected in skeletal muscle in response to insulin and contraction (Bruss et al., 2005; Kramer et al., 2006a). An AS160 mutant in which four of the phosphorylation sites are altered to alanine, but the Rab-GAP catalytic domain remains intact, markedly inhibited insulin-induced GLUT4 translocation in a dominant-negative manner in both adipocytes and skeletal muscle (Kramer et al., 2006b; Sano et al., 2003). Furthermore, depletion of AS160 by RNA interference increased the fraction of GLUT4 on the surface of unstimulated cells (Eguez et al., 2005; Larance et al., 2005). Collectively, insulin stimulates phosphorylation of AS160, leading to its dissociation from GLUT4 vesicles and the attenuation of the Rab-GAP activity, both of which may increase the active GTP-bound form of Rab proteins in GLUT4 vesicles (Figure 3). 14-3-3 proteins directly bind to AS160 in a phosphorylation-dependent manner (Ramm et al., 2006). Rab-GAP activity of AS160 may be negatively affected by the association with 14-3-3 proteins. TBC1D1, a close relative of AS160, is also a substrate of Akt2, and is characterized as a Rab-GAP that regulates GLUT4 traffic in response to insulin stimulation (Roach et al., 2007).

The TC10 Protein

In adipocytes, two independent signaling pathways are triggered by engagement of the insulin receptor (Kanzaki and Pessin, 2003). One is a PI3K-dependent pathway that leads to the activation of the downstream effector kinase Akt2, and the other is an adipocyte-specific pathway involving the Rho family small GTP-binding protein TC10 (Chiang et al., 2001; Kanzaki and Pessin, 2003; Figure 6). These two distinct signaling pathways act coordinately to elicit plasma membrane translocation of GLUT4 mediated by vesicle trafficking. The activation of TC10 takes place through the formation of a complex consisting of Cbl, CAP, CrkII, and C3G depending on tyrosine phosphorylation of Cbl in response to insulin stimulation (Chiang et al., 2001). This complex is localized specifically in caveolin-enriched lipid raft microdomains, in which TC10 is also localized. The specific localization in lipid

rafts may account for the specific function of the TC10 pathway in fully differentiated adipocytes. The activated TC10 in turn recruits the exocyst complex to the plasma membrane through the interaction between activated TC10 and the exocyst component Exo70 (Inoue et al., 2003; Figures 5 and 6). The exocyst complex is then required for the GLUT4 vesicle to be tethered to the appropriate sites of the plasma membrane before the final fusion step. Another mechanism by which TC10 enhances GLUT4 translocation involves Rab31 (Lodhi et al., 2007; Figure 6). A GEF for Rab31 called Gapex-5 is a binding partner of CIP4, which interacts with the GTP-bound active form of TC10. Insulin sequesters the CIP4/Gapex-5 complex away from its substrate Rab31 via the activation of TC10. The recruitment of the CIP4/Gapex-5 complex to the plasma membrane results in the failure of Rab31 activation, and thereby causes the escape of GLUT4 from the retention in the endosomal compartments (Lodhi et al., 2007). In addition, TC10 is involved in the regulation of actin cytoskeletal rearrangements like other Rho family GTP-binding proteins. Studies using constitutively activated and dominant-interfering mutants revealed that TC10 differentially regulates cortical and perinuclear actin polymerization in adipocytes (Kanzaki et al., 2002).

The Rac1 Protein

From an array of studies by the use of cultured myocytes and skeletal muscle, a pivotal role of reorganization of cytoskeletal structures in insulin-stimulated glucose uptake was clarified (Brozinick et al., 2004; Chiu et al., 2011; Kanzaki and Pessin, 2001; Tsakiridis et al., 1994).

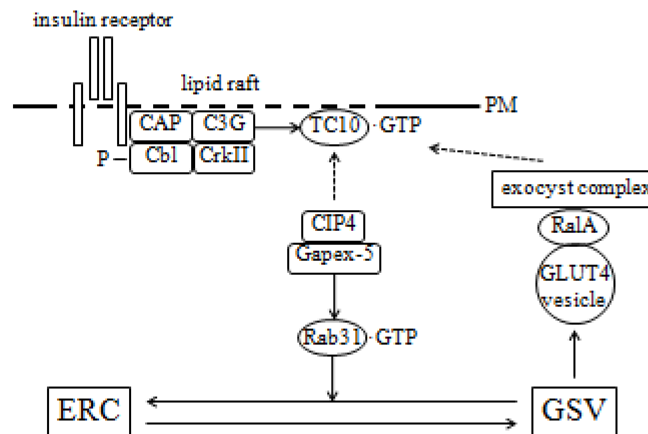

Figure 6. Role of TC10 downstream of the insulin receptor in adipocytes. Insulin triggers an adipocyte-specific TC10-mediated pathway in addition to the PI3K-dependent pathway. The GEF C3G forms a complex with the insulin receptor and other signaling proteins in response to insulin stimulation. TC10, when activated by C3G, recruits the GEF for Rab31 Gapex-5 to the plasma membrane through the interaction with its effector CIP4, thereby inhibiting the activation of Rab31. Inactivation of Rab31 permits GLUT4 trafficking from ERC to GSV. Moreover, TC10 tethers the GLUT4 vesicle near the plasma membrane through the interaction with the exocyst complex. ERC, endosomal recycling compartment; GSV, GLUT4 storage vesicle; PM, plasma membrane.

Reorganized microtubules and actin polymers serve as filaments to which GLUT4 vesicles are connected by kinesin (KIF3 and KIF5b) and myosin (Myo1c, Myo5a, and Myo5b) motor proteins, respectively (Bose et al., 2002; Imamura et al., 2003; Ishikura and Klip, 2008; Semiz et al., 2003; Yip et al., 2008; Yoshizaki et al., 2007). Considering that diverse Rho family GTP-binding proteins act as a regulator of cytoskeletal rearrangements in many types of cells, these proteins are likely to be involved in the regulation of GLUT4 translocation also in muscle cells.

Whereas TC10 has been implicated in insulin-stimulated GLUT4 translocation in adipocytes, TC10 may not play an important role in muscle cells (JeBailey et al., 2004). Instead, a role of another Rho family GTPase Rac1 in muscle cells has been proposed (Chiu et al., 2011; JeBailey et al., 2004; JeBailey et al., 2007; Khayat et al., 2000; Ueda et al., 2008). Actually, Rac1 activation was observed following insulin stimulation, and insulin-dependent GLUT4 translocation was almost completely abrogated by knockdown of Rac1.

Moreover, ectopic expression of an activated Rac1 mutant by itself induced GLUT4 translocation. By using muscle-specific *rac1* knockout mice, a pivotal role of Rac1 in inulin signaling is demonstrated in skeletal muscle as well (Ueda et al., 2010). Redistribution of GLUT4 in skeletal muscle was evaluated by immunostaining of an exofacial epitope-tagged GLUT4 reporter and immunogold electron microscopic analysis of endogenous GLUT4. Both assay clearly demonstrated that insulin-induced GLUT4 translocation to the skeletal muscle cell membrane was markedly reduced by muscle-specific *rac1* knockout.

Furthermore, ectopic expression of a constitutively activated Rac1 in mouse skeletal muscle in fact induced GLUT4 translocation as shown in the L6 cell line. Transgenic mice carrying a similar exofacial epitope-tagged GLUT4 reporter are also instrumental in visualizing GLUT4 translocation in response to contraction (Fazakerley et al., 2009; Schertzer et al., 2009). Recently, Sylow et al. (2013a) reported that insulin-stimulated glucose uptake was indeed lowered in *rac1* knockout mouse skeletal muscle. Importantly, *rac1* knockout mice showed decreased insulin and glucose tolerance, and glucose-stimulated plasma insulin concentrations in *rac1* knockout mice trended higher. Moreover, Rac1-dependent signaling was in fact impaired in insulin resistant human skeletal muscle. These findings strongly support the idea that Rac1 is a key regulator of insulin-dependent glucose uptake in skeletal muscle.

To my knowledge, FLJ00068 is a unique GEF that has been implicated in insulin-dependent Rac1 activation in muscle cells to date (Ueda et al., 2008). Overexpression of this GEF, but not other GEFs for Rac1 tested, enhanced insulin-stimulated Rac1 activation and GLUT4 translocation. A constitutively activated FLJ00068 mutant, when ectopically expressed, induced Rac1 activation and GLUT4 translocation. Moreover, knockdown of FLJ00068 largely inhibited insulin-dependent GLUT4 translocation. We recently observed that GLUT4 translocation to sarcolemma was indeed induced by constitutively activated FLJ00068 also in mouse skeletal muscle (unpublished results). Thus, FLJ00068 is considered to be a crucial Rac1 regulator in signaling that controls glucose uptake in skeletal muscle.

Insulin-induced Rac1 activation is mediated by PI3K (JeBailey et al., 2004; Ueda et al., 2008). However, precise mechanisms whereby PI3K induces the activation of Rac1 remain to be elucidated. Constitutively activated Rac1, when ectopically expressed, does not induce phosphorylation of Akt2, and insulin-stimulated Akt2 phosphorylation is not affected by muscle-specific *rac1* knockout (Ueda et al., 2008; Ueda et al., 2010). Therefore, Rac1 acts downstream or independently of Akt2. Our recent observations support the notion that Akt2

may be involved in PI3K-mediated Rac1 activation in muscle cells (Nozaki et al., 2013). Detailed mechanisms for Rac1 activation will be revealed in future studies.

Recently, a pivotal role of Rac1 in contraction-stimulated glucose uptake in skeletal muscle has been revealed (Sylow et al., 2013b). AMP-activated protein kinase-independent Rac1 activation was observed after exercise in both mice and humans. Furthermore, contraction-stimulated glucose uptake in mouse muscle was reduced by *rac1* knockout. Thus, Rac1 may play a central role in the regulation of glucose uptake in response to various physiological stimuli in skeletal muscle. Mechanisms for Rac1 activation, however, may differ depending on upstream signals. A GEF involved in contraction-stimulated Rac1 activation, for instance, remains obscure, and may be distinct from FLJ00068.

An important role of Rac1 in GLUT4 translocation in muscle cells is the regulation of actin cytoskeleton (Chiu et al., 2011). Cortical actin filaments serve as a track for GLUT4 vesicle transport (Loubéry and Coudrier, 2008). Myo5a and Myo5b act as a motor for GLUT4 vesicle trafficking along actin filaments in adipocytes and muscle cells, respectively (Ishikura and Klip, 2008; Yoshizaki et al., 2007). Additionally, cortical actin remodeling is required for tethering of GLUT4 vesicles near the plasma membrane and subsequent fusion events. In fact, Rac1 has been reported to participate in GLUT4 vesicle retention beneath the plasma membrane via actin cytoskeletal remodeling (Randhawa et al., 2008). The motor protein Myo1c may have a role as a link between the GLUT4 vesicle and actin filaments in the tethering step (Bose et al., 2002; Chen et al., 2007; Loubéry and Coudrier, 2008).

Ectopic expression of a constitutively activated Rac1 mutant induces GLUT4 translocation in muscle cultured cells and mouse skeletal muscle (Ueda et al., 2008; Ueda et al., 2010). Hence, it is likely that Rac1 participates not only in the regulation of cytoskeletal rearrangements, but also in other processes necessary for GLUT4 translocation although the mechanism underlying constitutively activated Rac1-dependent GLUT4 translocation remains to be clarified. Recently, it has been shown that the Ras family small GTP-binding protein RalA plays an important role in the control of GLUT4 translocation downstream of Rac1 in myoblast cells (Nozaki et al., 2012). Actually, constitutively activated Rac1 stimulated GTP loading on RalA and induced the redistribution of activated RalA to the membrane ruffling area, whereas knockdown of endogenous RalA abrogated constitutively activated Rac1-induced GLUT4 translocation. RalA may be responsible for the tethering step of Rac1-induced GLUT4 translocation in muscle cells because RalA regulates insulin-stimulated GLUT4 vesicle tethering to the plasma membrane through the interaction with exocyst and Myo1c complexes in adipocytes (Chen et al., 2007, see below). Detailed mechanisms whereby Rac1 regulates RalA activity and function, however, remain obscure, and will be clarified in future research.

In adipocytes, constitutively activated Rac1 does not induce GLUT4 translocation, and dominant-negative Rac1 does not affect insulin-induced GLUT4 translocation. Thus, in contrast to skeletal muscle, Rac1 may not participate in GLUT4 vesicle trafficking in adipocytes (Hou et al., 2006). However, a recent study highlights the role of Rac1 also in adipocytes. A Rac1-specific GEF termed P-Rex1, which is known to act downstream of PI3K, enhanced insulin-induced GLUT4 translocation and membrane ruffle formation (Balamatsias et al., 2011). This effect was indeed dependent on Rac1. Furthermore, knockdown of P-Rex1 inhibited insulin-induced glucose uptake.

Considering these results, Rac1 and its regulator P-Rex1 may be involved in insulin-dependent actin cytoskeletal remodeling and GLUT4 translocation to the plasma membrane

(Balamatsias et al., 2011). Interestingly, the *P-Rex1* gene has been mapped to a type 2 diabetes susceptibility locus, further supporting a role in insulin signaling (Lewis et al., 2010).

Many effector proteins for Rac1 have been identified in various types of cells. However, the effector that is responsible for transducing the Rac1 signal leading to glucose uptake remains unknown. Interestingly, the serine/threonine kinase PAK1, originally characterized as an effector for Rac1 and Cdc42, has been implicated in insulin-stimulated GLUT4 translocation in skeletal muscle (Wang et al., 2011). Actually, *PAK1* knockout mice exhibited peripheral insulin resistance coupled to this defect (Wang et al., 2011). Thus, it is possible that PAK1 plays a role in Rac1-mediated signaling in skeletal muscle, although Cdc42, another activator of PAK1, is not involved in insulin signaling (Ueda et al., 2008).

The RalA Protein

RalA resides in intracellular vesicles of exocytic compartments, participating in the regulation of polarized membrane transport and secretion, such as the basolateral delivery of membrane components in epithelial cells and synaptic vesicle trafficking in neuronal cells (Feig, 2003). RalA is also involved in the process of receptor-mediated endocytosis. The possibility that RalA participates in GLUT4 vesicle transport in insulin-responsive cells arose when the RalA function in vesicle trafficking was proposed. Actually, in adipocytes, RalA has been shown to regulate GLUT4 targeting to the plasma membrane in response to insulin (Chen et al., 2007). RalA is present in GLUT4 vesicles, and is activated following insulin stimulation. Activated RalA directly binds to Sec5 and Exo84 subunits of the exocyst complex, which mediates tethering of post-Golgi exocytic vesicles to the plasma membrane prior to membrane fusion (Chen et al., 2007; Moskalenko et al., 2002; Sugihara et al., 2002). The Myo1c motor protein, which connects the GLUT4 vesicle to the actin filament (Bose et al., 2002), also interacts with RalA. The binding of RalA to Myo1c is independent of the GDP/GTP status of RalA. Thereby, RalA directs the cooperation between Myo1c and the exocyst complex for GLUT4 vesicle transport along actin filaments and tethering to the plasma membrane (Chen et al., 2007).

A GTPase-deficient mutant of RalA, when ectopically expressed, increased glucose uptake in adipocytes and myocytes (Chen et al., 2011a; Nozaki et al., 2012). These results suggest that inactivation of RalA (hydrolysis of bound GTP to GDP) is not required for the disengagement of RalA from the exocyst complex before the fusion event. Alternatively, protein kinase C-mediated phosphorylation of the Ral-binding domain of Sec5 is reported to contribute to the dissociation of RalA (Chen et al., 2011a). Thus, not only the GDP/GTP cycle of RalA, but also the phosphorylation/ dephosphorylation cycle of its effector, are involved in the regulation of GLUT4 vesicle transport and tethering to the plasma membrane prior to membrane fusion. Mechanisms underlying insulin-dependent activation of RalA may be different between cell types. In adipocytes, inactivation of the Ral GAP complex through Akt2-catalyzed phosphorylation of the RGC2 subunit accounts for insulin-induced activation of RalA (Chen et al., 2011b). Although the mechanism for insulin-dependent activation of RalA in muscle cells remains incompletely understood, the involvement of Rac1 in insulin-dependent regulation of RalA may be a notable difference between muscle and adipose tissue (Nozaki et al., 2012).

The Arf Protein

Recently, the Arf subfamily member Arf6 has been implicated in insulin-stimulated GLUT4 recycling (Li et al., 2012). SecinH3 is a small molecule antagonist of the cytohesin family of brefeldin-A-insensitive GEFs for Arf GTP-binding proteins (Hafner et al., 2006). This compound was found to potently inhibit insulin-dependent cell-surface translocation of GLUT4 in adipocytes, suggesting the involvement of a cytohesin family GEF and its target Arf protein (Li et al., 2012). Through a series of experiments, the cytohesin family member Grp1 (also called cytohesin-3) and Arf6 have been identified as key players in insulin-induced GLUT4 vesicle formation as well as later steps of the specialized GLUT4 recycling pathway. Furthermore, phosphorylation of Grp1 by Akt has been proposed to be a mechanism for insulin regulation of Grp1.

Conclusion

Glucose uptake in adipose tissue and skeletal muscle is mediated by GLUT4 translocation from intracellular storage sites to the plasma membrane, and its precise regulation is critical for whole-body glucose homeostasis. Small GTP-binding proteins and their regulators play a pivotal role in many aspects of signal transduction that controls GLUT4 translocation as described above. Our understanding of the regulatory mechanisms, however, is still incomplete. Efforts to further delineate the molecular basis of the signaling mechanisms may provide clues for new potential therapeutic targets for diabetes.

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