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Signal transduction meets vesicle traffic: the software and hardware of GLUT4 translocation

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Klip A, Sun Y, Chiu TT, Foley KP. Signal transduction meets vesicle traffic: the software and hardware of GLUT4 translocation. *Am J Physiol Cell Physiol* 306: C879–C886, 2014. First published March 5, 2014; doi:10.1152/ajpcell.00069.2014.—Skeletal muscle is the major tissue disposing of dietary glucose, a function regulated by insulin-elicited signals that impart mobilization of GLUT4 glucose transporters to the plasma membrane. This phenomenon, also central to adipocyte biology, has been the subject of intense and productive research for decades. We focus on muscle cell studies scrutinizing insulin signals and vesicle traffic in a spatiotemporal manner. Using the analogy of an integrated circuit to approach the intersection between signal transduction and vesicle mobilization, we identify signaling relays ("software") that engage structural/mechanical elements ("hardware") to enact the rapid mobilization and incorporation of GLUT4 into the cell surface. We emphasize how insulin signal transduction switches from tyrosine through lipid and serine phosphorylation down to activation of small G proteins of the Rab and Rho families, describe key negative regulation step of Rab GTPases through the GTPase-activating protein activity of the Akt substrate of 160 kDa (AS160), and focus on the mechanical effectors engaged by Rabs 8A and 10 (the molecular motor myosin Va), and the Rho GTPase Rac1 (actin filament branching and severing through Arp2/3 and cofilin). Finally, we illustrate how actin filaments interact with myosin 1c and α-Actinin4 to promote vesicle tethering as preamble to fusion with the membrane.

GLUT4; insulin; Rab; Rac1; vesicle traffic

INSULIN STIMULATION OF GLUCOSE uptake into muscle is a fundamental response of the body, essential for nutrient utilization. Dietary glucose is primarily stored in skeletal muscle as glycogen, and therefore skeletal muscle is a primary regulator of glycemia in the fed state. At the core of this function lies the rapid (within minutes) mobilization of glucose transporters (GLUT4) to the surface of muscle fibers, effectively switching nutrient uptake from lipids to glucose. How this translocation of intracellular GLUT4 units is enacted has been the subject of intensive research for almost 30 years.

Being an integral membrane protein crossing the membrane 12 times, GLUT4 always lodges in lipid bilayers, and as a recycling protein it continuously cycles between the cell surface and intracellular storage sites. This involves an exquisite intracellular sorting mechanism through the endocytic apparatus that is regulated by insulin-derived signals. The insulin receptor at the muscle cell surface transmits intracellular signals that ultimately impinge on GLUT4-residing vesicles to promote their translocation to the cell surface. In its more essential analysis, this represents a case of signal transduction elicited by insulin that regulate vesicle traffic in a temporal and spatially precise fashion. In other words, insulin calls and GLUT4 responds.

How is this communication achieved and how is signaling information decoded into mechanical mobilization of vesicles? Instrumental in this analysis was the generation of muscle cell lines that respond to insulin and express tagged GLUT4 such that its presence that can be scored when at the surface and when within the cell. Rat L6 muscle cells are the best-studied insulin-responsive muscle cell line, and indeed insulin stimulation of Akt was first reported in these cells. To date, Akt phosphorylation represents the prototypical insulin response, although, as discussed in the following sections, it is only one of many steps in a highly structured signaling cascade that includes linear and bifurcated transmission of information.

By stably expressing *myc*-tagged GLUT4 into L6 myoblasts (L6GLUT4*myc* cell line), we have generated a cellular system in which we ascertained that GLUT4 cycles to and from the

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membrane; however, at steady state, a large portion of GLUT4 segregates in compartments separate from the recycling pathway. These cells also retain an intact insulin signaling machinery and hence constitute a highly useful cellular system to study how insulin signals impact on selective elements of the vesicle traffic machinery.

Work over two decades has shown that, in these L6GLUT4*myc* cells, insulin signaling toward GLUT4 is initiated by tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 (whereas IRS-2 is dispensable for this outcome) (22, 61). As in adipose cells, this activates a cascade of phosphorylation reactions beginning with activation of class I phosphatidylinositol (PI) 3-kinase (PI3K) to generate the phospholipid PI3,4,5-P_3; engagement of both PI-dependent kinase (PDK) and mTORC2 to phosphorylate the serine/threonine kinase Akt (particularly isoform Akt2); and phosphorylation of the Akt substrate of 160 kDa (AS160) (14, 29, 51, 62, 67). In parallel, in both muscle and fat cell lines, insulin triggers signals that regulate the actin cytoskeleton, and each of the elements in these signaling cascades has been shown to be required for insulin-dependent GLUT4 translocation (64, 65).

Here we review how these signals (the "software") transmit information to downstream effectors that mediate mechanical work (the "hardware") to mobilize GLUT4 vesicles to the plasma membrane. This review is presented from the perspective of findings in L6GLUT4*myc* cells, with pertinent reference to observations made in mature skeletal muscle fibers and adipocytes in culture. The nature of the intracellular compartment(s) harboring GLUT4 is the subject of active investigation and will not be reviewed here. Readers are referred to excellent reviews on the state of that knowledge (4, 18, 53).

Bifurcation Downstream of PI3K: Akt and Rac

As shown in Fig. 1, PI3K signaling bifurcates leading to activation of Akt and actin remodeling, respectively. The cytoskeleton-regulating cascade is typified by activation (i.e., GTP loading) of Rho family G proteins, which in muscle cells are predominantly Rac1 (26, 27) and in adipocytes also include TC10 (11). Rho family GTPases are switch molecules and are therefore prototypical software of insulin signaling.

Rac1 is activated in response to insulin in muscle cells and mature skeletal muscle, and, notably, mice with muscle-specific deletion of the Rac1 gene develop insulin resistance and have reduced muscle glucose uptake in response to the hormone (57, 66). Rac1 activation requires active PI3K (26), and in muscle and fat cells it is likely the result of PI3,4,5-P_3-dependent activation of selective GDP/GTP exchange factors (GEFs). In addition, there may be regulation at the level of GTPase-activating proteins (GAPs) and GDP dissociation inhibitors.

Activated Rac1 leads to cortical actin branching, mediated by the Arp2/3 complex. Therefore, this can be considered to be a mechanical hardware in the pathway leading to GLUT4 mobilization. Enabling Arp2/3 to promote actin branching leads to the formation of dynamic membrane ruffles supported by branched actin filaments. In turn, the increase in polymerized actin sets up a feedback reaction as actin filaments activate phosphatases that dephosphorylate and thereby activate the actin filament-depolymerizing enzyme cofilin (12). This results in an iterative cycle of actin branching and depolymerization,

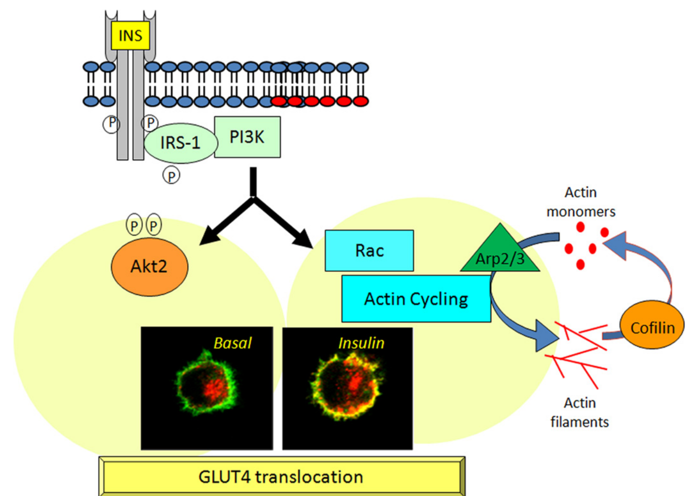

Fig. 1. Insulin (INS) signal bifurcation leading into GLUT4 translocation. Downstream of phosphatidylinositol 3-kinase (PI3K) in the insulin signaling cascade, the relay of information segregates into 2 independent arms typified by the activation of Akt and Rac, both of which lead to GLUT4 translocation to the membrane. Rac stimulates the continuous actin cycling marked by actin branching and filament depolymerization, controlled by Arp2/3 and cofilin, respectively. The insulin-elicited dynamic actin structures likely serve to tether and concentrate GLUT4 vesicles in the vicinity of the plasma membrane. The fluorescence image shows GLUT4 vesicles stained in red and the plasma membrane stained in green, in suspended, rounded-up L6GLUT4*myc* myoblasts. Accordingly, GLUT4 at the plasma membrane appears yellow. IRS-1, insulin receptor substrate-1.

both being required for effective insertion of GLUT4*myc* into the plasma membrane (Fig. 1). In addition, insulin-induced activation of Rac1 also leads to activation of the serine/threonine kinase Pak1 (27, 57). How this dynamic cycle of mechanical actin filament branching and depolymerization directly promotes GLUT4 translocation is still being investigated, but evidence supports contributions to GLUT4 vesicle concentration/tethering beneath the membrane (39, 55, 59, 64, 68) and potential changes in membrane tension (5). The hardware constituted by the dynamic cortical actin may also contribute to positioning of software elements of the phosphorylation cascade (32, 37).

Interestingly, the actin cytoskeleton-regulating cascade (typified by Rac1 activation and actin branching) is independent and dispensable from the phosphorylation cascade downstream of PI3K (typified by Akt activation). Specifically, inhibition of Akt is without effect on Rac1 activation or signaling to the cytoskeleton in muscle cells and skeletal muscle (13, 58). Conversely, inhibiting the cytoskeleton-regulating cascade by downregulating Rac1 expression or activity, or by preventing actin polymerization, is largely inconsequential on Akt activation by insulin (12, 27). However, inhibition of either PI3K-dependent cascade alone inhibits GLUT4*myc* translocation to the cell surface in response to insulin (12, 27, 58). Nonetheless, superactivation of Rac1 to levels far exceeding those elicited by insulin, such as by overexpression of constitutively active Rac1 mutants, can signal back to PI3K and thereby activate Akt in the absence of insulin. In these conditions, GLUT4 translocation is promoted to furnish GLUT4*myc* at the cell surface in an insulin-independent fashion, raising the concept that acute, intense yet controlled Rac1 activation might provide a strategic avenue to overcome insulin resistance when it arises from defective activation of steps upstream of PI3K (13).

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AS160: the "Brake" in Insulin Signaling

Although the insulin-dependent activation of Akt has been known for almost two decades, only lately have downstream events unraveled. Akt has diverse targets, but leading to GLUT4 translocation what stands out is the aforementioned AS160, a GAP toward small G proteins of the Rab family. Lienhard and collaborators (34, 46) first described this response and laid out the important concept that insulin-dependent, Akt-mediated phosphorylation of AS160 eliminates the GAP activity, effectively allowing activation of the purported target Rab G proteins to prevail. Indeed, AS160 mutants that cannot get phosphorylated by Akt, such as AS160-4A, function in dominant fashion as constitutively active GAPs, and this strategy prevents insulin-dependent GLUT4 translocation in adipocytes and muscle cells (10, 24, 46, 55). Conversely, silencing the expression of AS160 via siRNA in both muscle and adipose cells causes a gain in surface GLUT4 in the absence of insulin. Hence, one can conceptualize AS160 as a constitutive brake in insulin signal transmission, which is physiologically released upon ignition of the Akt phosphorylation software (17, 25).

Rab Family GTPases: Insulin-Regulated "Molecular Switches" that Engage Mechanical Effectors

Like Rho-GTPases in the actin polymerization cascade, Rab G proteins are molecular switches that, in their GTP-loaded form, transmit signals to downstream effectors and hence are essential elements of the insulin signaling-dependent software. Notably, Rab GTPases are quintessential regulators of intracellular vesicle traffic through engagement of mechanical effectors responsible for vesicle budding, mobilization, and fusion (49). Hence, one can conceptualize that effectors engaged by activation of Rab G proteins would include some that enact mechanical work and constitute further hardware needed for vesicle budding and fusion reactions. A classical example of Rab G protein engagement of mechanical effectors is the formation of a complex between Rab27-GTP and the molecular motor myosin Va (MyoVa) mediated by melanophilin, and a direct complex between Rab11 and MyoVb (23, 42). Given that the human genome encodes for at least 60 Rab GTPases (52), it becomes paramount to identify those that are specific targets of the GAP activity of AS160, since those would be expected to become active in response to insulin.

Rab8A, Rab10, Rab13: Molecular Switches of Insulin Signaling Downstream of Akt

The identification of Rab molecules activated by insulin is one of the most exciting discoveries of recent years, as they become the last step in the relay from the software to the hardware mobilizing GLUT4. In vitro, a number of Rab proteins are targets of the TBC domain (GAP activity domain) of AS160 (34), specifically Rabs 2A, 8A, 10, and 14, and all but Rab2A are detected in membranes enriched in GLUT4 isolated from mouse 3T3-L1 adipocytes (33, 34). Further studies in those cells identified Rab10 as necessary for GLUT4 translocation, with additional minor contribution reported for Rabs 8A and 14, possibly each acting at distinct steps in vesicle traffic (48). More recent work positions Rab14 in the endocytic transit of GLUT4 en route to the insulin-responsive compartment (41, 44). Interestingly, in rat muscle cells Rab8A is required for insulin-dependent GLUT4 translocation whereas Rab10 is dispensable (24, 25, 55, 56). These differences were first noted in experiments where the expression of individual Rab GTPases was silenced via cognate siRNAs. Because knockdown of Rab10 in adipocytes and of Rab 8A in muscle cells reversed the gain in surface GLUT4 caused by silencing AS160, these GTPases were positioned as targets of AS160 in the respective cells in the absence of insulin. By this same experimental paradigm, Rab14 was positioned as a target of TBC1D1, an AS160-related GAP (25).

In addition to Rab8A, muscle cells also engage Rab13 in the translocation of GLUT4 vesicles, as evinced by loss of this insulin response in muscle cells depleted of Rab13. Rabs 8A, 10, and 13 belong to a cluster of highly related Rab GTPases, but Rab13 had not been investigated in the in vitro studies examining AS160 targets. That Rab8A and Rab13 are bona fide targets of AS160 was shown by the restoration of insulin-dependent GLUT4 translocation upon overexpression of Rab8A or Rab13 in muscle cells also overexpressing the constitutively active AS160-4A mutant (24, 55). This functional complementation that rescues insulin action from the block imposed by AS160 that cannot be inactivated demonstrates that Rab8A and Rab13 are indeed insulin-dependent signals acting downstream of AS160 (Fig. 2). A similar complementation of insulin action was more recently reported for Rab10 in adipose cells (10).

The above studies illustrate that Rab 8A and 13 in muscle cells, and Rab10 in adipocytes, are necessary and under certain conditions sufficient to evoke insulin-dependent GLUT4 translocation. Although implied, these experiments did not show that insulin leads to activation (GTP loading) of these GTPases. This requirement has been fulfilled for Rab8A and Rab13 in muscle cells, which can be photo-covalently labeled by photoactivatable GTP (55). Rab8A activation occurred by 2 min of addition of insulin, followed by Rab13 activation by 5 min. In contrast, Rab10 was not activated in response to insulin in muscle cells, and it is so far unknown whether the hormone leads to activation of this G protein in any other cell type.

Because AS160 is a GAP that promotes its cognate Rab GTPases to hydrolyze GTP and remain GDP loaded (i.e., inactive), the result of its insulin-dependent phosphorylation and possibly departure from GLUT4 vesicles would allow the GTP-loaded form of its target Rabs to prevail. However, this requires that GTP loading would have occurred in the first place. Clearly, this step must be catalyzed by dedicated GEFs, and although these may be constitutively active it is also very likely that these GEFs are subjected to regulation in response to insulin. Accordingly, the search for the GEFs for Rab8A, Rab10, and Rab13 is highly relevant. Recently, GEFs for a number of Rab GTPases have been identified (2) and from those DENND4A-C were found to be highly preferential for Rab10 (71). Interestingly, knocking down DENND4C in adipocytes prevented the gain in surface GLUT4 signaled by insulin (47). Next, it will be important to find out whether its activity is regulated. Known GEFs for Rab8A include Rabin8/Sec2p and DENND1c, and ongoing work should identify which of these are engaged in the insulin-regulated activation of Rab8A and how. Identification of GEFs for Rab13 is still largely outstanding. The preferential participation of Rabs 8A and 13 in rat muscle cells and Rab10 in mouse adipocytes is

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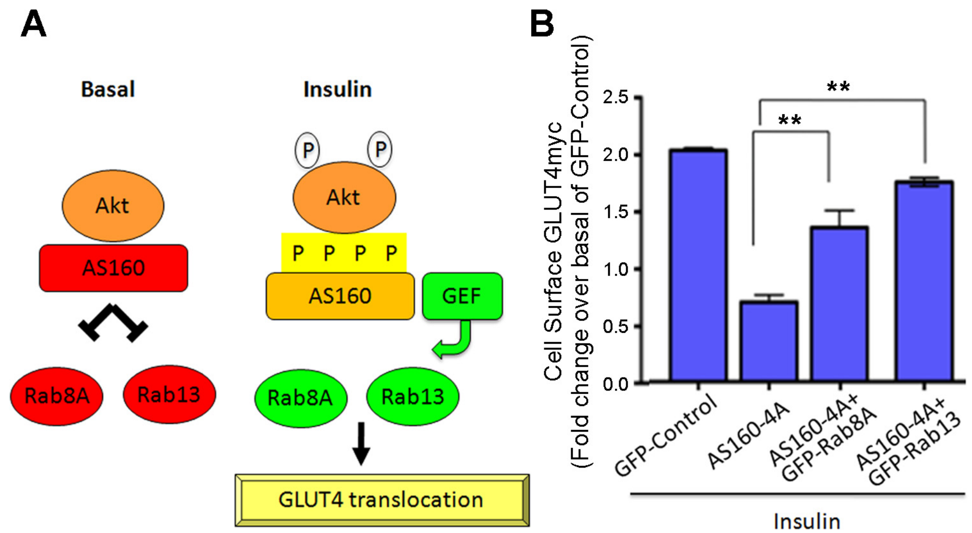

Fig. 2. Signals leading to Rab activation downstream of AS160 in muscle cells. *A*: Rab8A and Rab13 lie downstream of AS160. In the basal state, the GTPase-activating protein (GAP) activity of AS160 suppresses Rab8A and Rab13. Upon insulin stimulation, Akt phosphorylates AS160 to inhibit its GAP activity. This enables the GDP/GTP exchange factor (GEF)-mediated activation of downstream Rabs to prevail, as required for GLUT4 translocation. *B*: Rab8A and Rab13 overexpression rescue GLUT4 translocation from inhibition by the constitutively active AS160-4A. Constitutively active AS160 (AS160-4A) impairs insulin-mediated GLUT4 translocation. However, this defect can be overcome by overexpression of Rab8A or Rab13, reinforcing the idea that these Rabs act downstream of AS160. Shown are the mean ± SE from three independent experiments (** $P < 0.01$).

intriguing; more will be learned about the differential roles when their complements of downstream effectors are identified.

Myosin Va: a Processive "Molecular Motor" Propelling GLUT4 Vesicle Traffic

As mentioned above, Rab GTPases transmit their signal by engaging effectors that often enact mechanical work propelling vesicle traffic. By this paradigm, Goldenring and collaborators had identified that Rab8A and Rab10 interact with myosins Va, Vb, and Vc, molecules that form homodimers that bind to actin filaments through their head group and bind the GTPases at their COOH-terminal tail domain (42). MyoV molecules belong to the class of processive molecular motors given their ability to propel cargo along actin filaments, powered by the energy of their ATPase intrinsic activity (50). Of these, MyoVb binds Rabs 8A and 10 as well as Rab11, a G protein involved in vesicle recycling that is not an AS160 substrate. Regarding MyoVa, differential splicing results in two versions, and only the +D form containing the D exon can bind Rabs 8A and 10 (42).

The participation of myosin V molecular motors in GLUT4 traffic was first determined in 3T3-L1 adipocytes, when MyoVa was found to be phosphorylated by Akt and required for insulin-stimulated GLUT4 translocation (72). However, a mechanistic link to AS160 or Rab proteins was not established until it was shown in muscle cells that overexpression of a fragment of MyoVb encoding the Rab8A and Rab10 binding domain aborted insulin-dependent GLUT4 translocation (39). This fragment acted as a scavenger for these Rab GTPases, preventing their participation in GLUT4 traffic. Subsequently, MyoVa was identified as a partner of Rab10 in adipocytes and overexpression of the MyoVa COOH-terminal fragment interfered with the behavior of GLUT4 vesicles within 100 nm of the plasma membrane (10). This zone was imaged by total internal reflection fluorescence (TIRF) microscopy. More recently, it was clarified that while the exogenous MyoVb fragment acted as a Rab scavenger, in the intact muscle cell only MyoVa is expressed. Notably, in these cells MyoVa functions to allow GLUT4 vesicles to reach the cell periphery once engaged by Rab8A in an insulin-dependent fashion, acting at a step prior to GLUT4 vesicle arrival at the TIRF-imaged submembrane zone (56). In Chinese hamster ovary cells expressing insulin receptors used as a test system, binding of Rab8A to MyoVa was enhanced by insulin, whereas binding of Rab10 was not similarly regulated. In this study, a cluster of distinctive amino acids was identified to bind Rab8A but not Rab10, and abrogation of these residues in MyoVa transfected into L6GLUT4*myc* cells prevented insulin-dependent GLUT4 translocation (56).

Hence, Rab8A in rat muscle cells and Rab10 in mouse adipocytes engage MyoVa, but so far the functions appear to pertain to events occurring, respectively, before or within 100 nm of the plasma membrane. In any case, these studies cement the participation of MyoVa as a downstream effector of Rab proteins that is activated in response to insulin-dependent phosphorylation of AS160 by Akt (Fig. 3). Potentially, in muscle cells Rab13 might fulfill roles in the vicinity of the plasma membrane that in adipose cells are mediated by Rab10. However, Rab13 does not bind MyoVa and hence different effectors must carry out the Rab13 signal. Recent work identifies the protein linker MICAL-L2 as a Rab13 effector in epithelial and neural cells (35, 45, 60), and the positioning of both of these proteins in the submembrane region, as opposed to the more perinuclear location of Rab8A, suggests they may regulate steps in vesicle traffic distinct from those regulated by Rab8A. This distinctive localization may work in conjunction with the temporal sequence of activation of Rab8A in response to insulin, which precedes activation of Rab13 (55).

Myosin 1c: a Nonprocessive Molecular Motor Engaged in GLUT4 Vesicle Tethering to Actin Filaments

MyoVa is not the only myosin molecule participating in insulin-dependent GLUT4 vesicle traffic. Indeed, the monomeric Myo1c was found by the groups of Czech, Saltiel, and James to be an element required for GLUT4 translocation (6, 9, 21, 70), but its precise step of action was not identified. Recent studies in muscle cells reveal that Myo1c functions to tether GLUT4 vesicles at the TIRF-imaged zone (5). Accordingly, the insulin-induced immobilization in this region observed in response to insulin was lost in cells depleted of Myo1c or expressing mutants of Myo1c unable to link to actin filaments.

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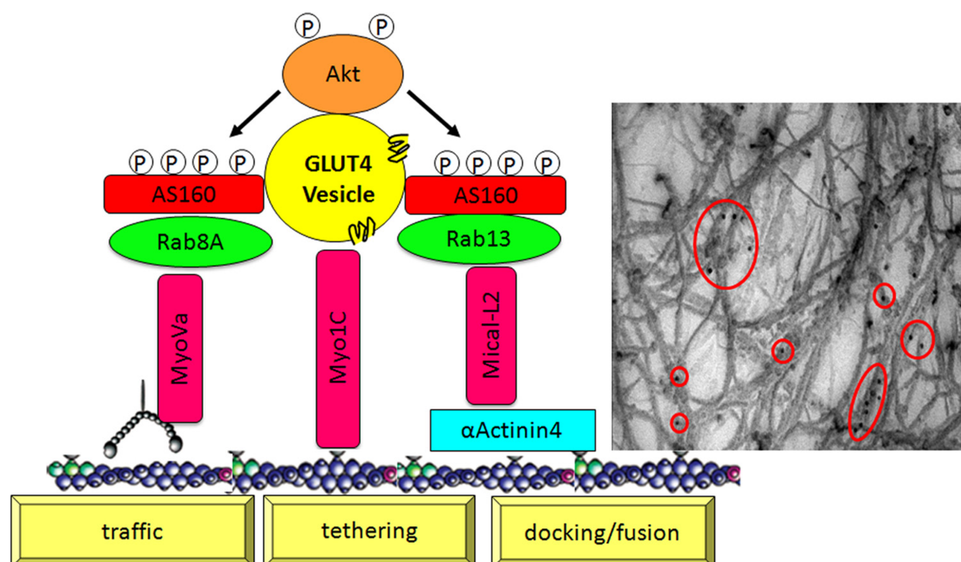

Fig. 3. Insulin signals regulate distinct steps in GLUT4 vesicle traffic. Activated Rab8A interacts with the processive motor myosin Va (MyoVa), which mobilizes GLUT4 vesicles toward the plasma membrane potentially aided by procession along actin filaments. GTP-loaded Rab13 can engage the adaptor protein Mical-L2, possibly to deliver GLUT4 vesicles within reach of the cortical actin structures possibly via its interaction with α-Actinin4. Additionally, tethering of GLUT4 vesicles to actin can be achieved through direct interaction between the vesicle and Myo1c. These proposed mechanisms for traffic, tethering, and docking of GLUT4 vesicles engage dynamic actin filaments. The presence of GLUT4 along actin filaments is illustrated by scanning electron microscopy detection of immunogold-labeled GLUT4*myc* (encircled in red) along the actin cytoskeleton beneath the plasma membrane Image from supplemental material of Randhawa et al. (39).

Moreover, Myo1c was visualized on GLUT4 vesicles in the TIRF-imaged zones and tracked with immobile more than mobile vesicles (5).

Given the prominence of actin filaments in the cortical region, a working model has emerged whereby GLUT4 vesicles arriving at the cell periphery likely become tethered to the dynamic cortical actin filaments. This may enable a transient collection of vesicles, which may be subsequently released in quantal spurs upon dissolution of the actin mesh through its iterative depolymerization-branching cycles (Figs. 1 and 4). This model integrates the Rac-driven cycling of cortical actin with the Akt-driven engagement of Rab molecules that furnish the cortical region with GLUT4 vesicles. Interestingly, Myo1c appears to not obey regulation by either Rac or Akt, instead passively linking incoming GLUT4 vesicles (driven by an Akt-AS160-Rab signaling axis and motorized by MyoVa) with the dynamic cortical actin mesh (which, as mentioned earlier, is driven by a Rac1-Arp2/3 signaling axis mechanized by actin filament branching and severing cycles). Hence, this model integrates the merging of insulin-derived software with its effector hardware molecules.

GLUT4 vesicle tethering to dynamic actin filaments is thus mediated by Myo1c. In addition, another molecule contributes to GLUT4 vesicle retention in the cortical zone, the actin filament-binding protein α-Actinin4 (ACTN4). This filamentous protein was identified as a direct GLUT4 protein partner in response to insulin, through a proteomic analysis based on the screen of insulin-dependent GLUT4 partners using stable isotope-linked amino acids (20). ACTN4 may thus introduce molecular selectivity in the above model of GLUT4 vesicle tethering onto actin filaments. Silencing the expression of ACTN4 did not affect insulin-induced Akt activation or actin remodeling, but it prevented GLUT4 vesicle collection within the filamentous subcortical actin mesh (59). Hence, ACTN4 appears to hook vesicles to the cortical actin filaments.

Vamp2, Syntaxin4, and SNAP23: the "Fusion Machinery" for GLUT4 Membrane Insertion

The collection of GLUT4 vesicles in the TIRF-imaged zone begs analysis of the subsequent steps leading to vesicle docking at the membrane and productive fusion. A body of work examining elements of this step concluded earlier on that VAMP2 on the GLUT4 vesicle and syntaxin4 and SNAP23 on the plasma membrane are required for GLUT4 vesicle fusion in muscle and adipose cells (19, 30, 40). This work is extensive and will not be reviewed in detail here. A critical question is whether this step occurs passively once GLUT4 vesicles concentrate and tether beneath the membrane, or whether active

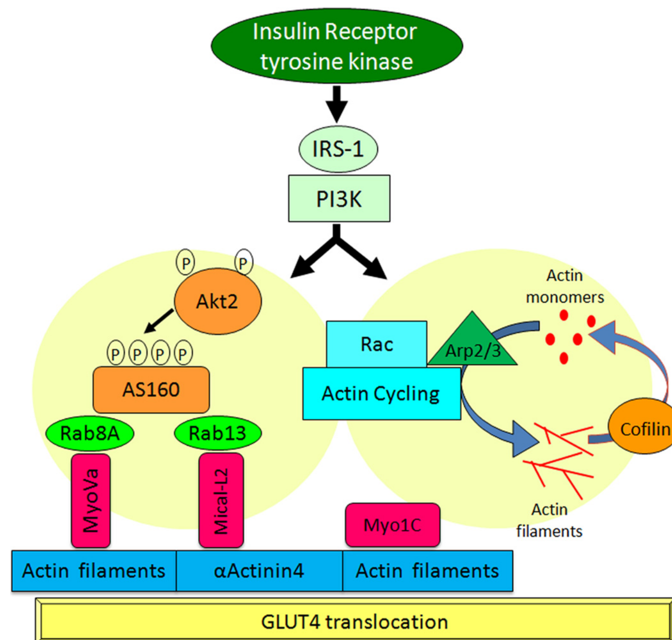

Fig. 4. Consolidated view on the software and hardware molecules that drive insulin-stimulated GLUT4 translocation in muscle cells. Insulin signaling bifurcates into the Rac and Akt pathways. The software signal Rac, a molecular switch, controls the actin cytoskeletal hardware to provide a tethering/docking zone beneath the plasma membrane. The software signals Akt and AS160 connect to the molecular switches Rab8A and Rab13, which in turn engage the hardware molecules: the motor MyoVa, the linker Mical-L2, and the tether Myo1C. These in turn connect to the actin hardware. The intricate coordination between the "software" and "hardware" results in insulin-responsive GLUT4 translocation to the membrane of muscle cells.

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insulin-dependent signaling also impinges on the actual fusion machinery. Most studies point to a regulation of syntaxin4 by Munc18 phosphorylation (8, 31, 63), although input at the level of VAMP2 was also proposed (7).

An Additional Regulator: Cytosolic Ca^{2+}

The model presented in Fig. 4 represents the signals downstream of class I PI3K known thus far and proposes a logical progression from the insulin receptor through tyrosine, lipid and serine/threonine kinases down to Rho and Rab GTPases, myosin motors and actin branching and depolymerization.

Beyond these signals, other pathways have emerged to provide further input. In keeping with the focus of this review to insulin signaling toward GLUT4 in muscle cells, it is important to highlight the growing evidence for the participation of Ca^{2+}-derived signals. In particular, in primary myotubes, L6 myotubes, and neonatal cardiomyocytes, insulin causes a sharp and transient increase in cytosolic Ca^{2+} (15, 16). This Ca^{2+} spike originates from the opening of both ryanodine-sensitive and IP_3-gated intracellular Ca^{2+} channels. The evidence thus far indicates that these channels, respectively, respond to signals initiated by the insulin receptor-mediated increases in H_2O_2 and by PI3K-$\gamma \rightarrow$ phospholipase C activation (16, 28). The readout of the cytosolic Ca^{2+} spike is still under investigation, but inhibition of each of the elements of the pathway prevents GLUT4 insertion in the membrane. Whether this is the result of prevention of other signals, of vesicle mobilization, or of fusion with the membrane remains to be determined.

Closing Remarks

Insulin signals act as molecular relays causing transient activation of molecular switches that in turn engage a number of mechanical effectors of vesicle traffic. This constitutes an integrated circuit of software and hardware activated by a plasma membrane-bound insulin receptor. We have illustrated the exquisite regulation of GLUT4 traffic at diverse points in its intracellular itinerary within muscle cells. Regulation of specific steps has gained momentum and recognition, mostly pertaining to GLUT4 vesicle mobilization, collection in the TIRF-imaged zone, and possibly promotion of vesicle fusion. The rate-limiting steps remain to be determined.

Interesting new concepts have emerged, such as the prominence in insulin signaling of small G proteins regulating actin dynamics and vesicle traffic, and engagement of molecular motors that both propel and tether vesicles in their journey to the plasma membrane. For the most part, studies in muscle and adipose cells reinforce similar concepts and often have been synergistic in the effort to generate working models of GLUT4 translocation.

Clearly, selective steps still require elucidation, for example identifying the GEFs for Rac and Rabs and identifying whether Munc18c is regulated by one or more inputs, such as the insulin receptor and protein phosphatase 1B (2a, 8), synip (36), and/or tomosyn (69). Equally important will be to decipher where does GLUT4 insert on the membrane and whether hotspots are defined by the cytoskeleton and/or caveolar elements (1, 43, 54). To date, caveolae have been shown to be important for insulin signaling and GLUT4 endocytosis in adipocytes, but their possible participation in GLUT4 exocytic traffic requires further exploration.

The critical mechanisms and signals controlling GLUT4 retrieval from the plasma membrane and intracellular sorting are still being investigated and have been omitted from this review but represent an exciting and intense area of study. Importantly, how GLUT4 sorts to and is sequestered in an insulin-sensitive compartment is being unraveled (18a). In this context, the GLUT4-binding protein TUG (3) and the t-SNARE Syntaxin6 (18a, 38) have emerged as key elements controlling GLUT4 vesicle intracellular retention and acquisition of insulin responsiveness, respectively.

Future studies should reveal whether and how selective steps in this integrated circuit representing the coalescence of signal transduction and vesicle traffic fail in nutritional and inflammatory conditions causing insulin resistance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.K. conception and design of research; A.K., Y.S., T.T.C., and K.P.F. analyzed data; A.K., Y.S., T.T.C., and K.P.F. interpreted results of experiments; A.K., Y.S., and T.T.C. prepared figures; A.K. drafted manuscript; A.K., Y.S., T.T.C., and K.P.F. edited and revised manuscript; A.K., Y.S., T.T.C., and K.P.F. approved final version of manuscript; Y.S., T.T.C., and K.P.F. performed experiments.

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