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Biogenesis and regulation of insulin-responsive vesicles containing GLUT4

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Abstract

Insulin regulates the trafficking of GLUT4 glucose transporters in fat and muscle cells. In unstimulated cells, GLUT4 is sequestered intracellularly in small, insulin-responsive vesicles. Insulin stimulates the translocation of these vesicles to the cell surface, inserting the transporters into the plasma membrane to enhance glucose uptake. Formation of the insulin responsive vesicles requires multiple interactions among GLUT4, IRAP, LRP1, and sortilin, as well as recruitment of GGA and ACAP1 adaptors and clathrin. Once formed, the vesicles are retained within unstimulated cells by the action of TUG, Ubc9, and other proteins. In addition to acting at other steps in vesicle recycling, insulin releases this retention mechanism to promote the translocation and fusion of the vesicles at the cell surface.

Introduction

Several examples of regulated, non-secretory exocytosis of membrane vesicles have been described [1]. In vertebrates, the exocytic translocation of various membrane proteins occurs in response to hormones and other extracellular stimuli, providing a mechanism for the regulation of systemic physiology. One of the best-studied examples of this process is the insulin-stimulated translocation of GLUT4 glucose transporters [2–4]. A 12-transmembrane protein, GLUT4 is the predominant glucose transporter present in fat and muscle. Insulin regulates glucose uptake by controlling the number of transporters at the cell surface, and acts within minutes to mobilize GLUT4 from intracellular stores. Because this action is compromised during the development of type 2 diabetes, much work has been carried out to understand both the insulin signaling and GLUT4 trafficking pathways that mediate this process.

Multiple insulin signaling pathways have been implicated in GLUT4 regulation, and may intersect with GLUT4 trafficking pathways at distinct steps. Greatest attention has been given to signaling through Akt2 to AS160/TBC1D4 and TBC1D1, Rab GTPase-activating proteins that direct GLUT4 targeting in fat and muscle, respectively [5]. Other signaling

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pathways that have been implicated involve atypical protein kinase C isoforms and the Rho-family GTPase, TC10 α [6,7]. These pathways are reviewed elsewhere [3–5].

Here, we focus on protein trafficking mechanisms that regulate GLUT4. In particular, we summarize recent work that has led to improved understanding of how GLUT4 and other proteins assemble in insulin-responsive vesicles (IRVs), how these vesicles are retained intracellularly in unstimulated cells, and how they may be mobilized by insulin.

Protein sorting into IRVs

Immunoelectron microscopy of adipose and skeletal muscle cells as well as biochemical data demonstrate that under basal conditions, up to 75% of total intracellular GLUT4 is localized in small (50 - 80 nm in diameter, ca. 80S) vesicles and short tubules [2,3]. The rest of the transporter is present in large, rapidly sedimenting intracellular membranes that likely represent endosomes and trans-Golgi network (TGN) structures. Small GLUT4-containing vesicles do not represent a homogeneous compartment and include at least two vesicular populations: the insulin-responsive vesicles, or IRVs, that are translocated to the plasma membrane with virtually 100% efficiency, and ubiquitous intracellular transport vesicles that are not recruited to the plasma membrane by insulin. In adipocytes, the latter vesicles are marked by the presence of cellugyrin [8,9], a 4-transmembrane protein with as yet unknown physiological functions that represents a ubiquitous homologue of synaptogyrin, a major constituent of synaptic vesicles.

A systematic analysis of highly purified IRVs was recently performed by Jedrychowski *et al.* [9]. Results of this study confirmed that GLUT4, IRAP and sortilin, the proteins that had previously been found in the total preparation of small GLUT4-vesicles, were present in the IRVs in multiple copies. In addition, Jedrychowski *et al.* found that the low density lipoprotein receptor-related protein 1 (LRP1) also resides in the IRVs. LRP1 has long been known to undergo insulin-dependent translocation to the plasma membrane in adipose cells, but escaped previous proteomic analysis of GLUT4-vesicles, likely, because of its enormous size (4544 amino acid residues). Finally, the IRVs contain VAMP2 that represents the v-SNARE in compartment [10]. Other proteins may also reside in the IRVs as relatively minor components.

Each round of insulin stimulation leads to the plasma membrane translocation of all IRV proteins. As there is a low occurrence of kiss-and-run events [11], each IRV protein is likely to be internalized from the plasma membrane at its own rate. This means that functional IRVs must be somehow re-assembled from the individual components after each translocation event, i.e. thousands and tens of thousand times throughout the life cycle of a fat or skeletal muscle cell. The mechanism of IRV assembly should work with high fidelity, as malfunctioning of this process may decrease the number of the IRVs in the cell or lead to the formation of less insulin-sensitive vesicles and, eventually, to insulin resistance. So, how do GLUT4, IRAP, sortilin and LRP1 find each other in the cell to form a unique, highly specialized vesicular carrier?

From the plasma membrane, IRV proteins are internalized into sorting endosomes and eventually reach a syntaxin-6/16 -positive perinuclear compartment. This perinuclear compartment likely includes a sub-domain of the TGN as well as recycling endosomes [2,3]. In human skeletal muscle, retrograde trafficking of GLUT4 from sorting endosomes to the TGN is an essential step in the “GLUT4 pathway” and may involve syntaxin-10 [12]. In humans, this step is mediated by the specific isoform of clathrin heavy chain, CHC22 [13]. Interestingly, neither CHC22 nor syntaxin-10 is expressed in mice; this may contribute to a relatively lesser input of skeletal muscle in overall glucose homeostasis in mice versus humans.

From a biochemical standpoint, the perinuclear compartment represents a mixture of IRVs and large, rapidly sedimenting “donor” membranes [14,15] that exist in a dynamic equilibrium [16–18]. Although the resolution of conventional fluorescence microscopy cannot distinguish the donor membranes from small vesicles, these can be readily separated by biochemical fractionation [15].

GLUT4, IRAP and sortilin are targeted to the perinuclear donor membranes by their cytoplasmic domains [14,15,19,20]. Upon arriving in this compartment, these proteins can redistribute to the IRVs by mass-action [21]. Specifically, GLUT4, IRAP, sortilin and a newly discovered IRV component, LRP1, can interact with each other via luminal domains [9,14,15,20]. Thus, mutual luminal interactions may bring these proteins together in the donor membranes and facilitate active protein sorting into the IRVs.

Biogenesis of IRVs

The formation of membrane vesicles is driven by protein coats that are recruited to specific sites on donor membranes by adaptor proteins. The latter associate with donor membranes through multiple interactions with the cytoplasmic domains of cargo proteins, Arf-GTP and phosphatidylinositol phosphates [22]. Formation of the IRVs on intracellular donor membranes requires clathrin coats [23,24] and GGA adaptors [25,26]. The only protein component of the IRVs that is known to interact with GGA is sortilin, which has a conventional DXXLL GGA-binding motif [27]. However, since sortilin can interact with GLUT4, IRAP and LRP1 in the vesicular lumen, it may function as a transmembrane scaffold by “gathering” the major IRV component proteins in a large oligomeric complex. Then, sortilin, GLUT4, IRAP and LRP1 can partition into the vesicular fraction as a single entity using the GGA-dependent vesicle budding machinery [14].

GGA adaptors may not be sufficient for the formation of the IRVs. Another adaptor protein that plays a major role in the formation of these vesicles is ACAP1, which interacts with the central cytoplasmic loop of GLUT4 and recruits clathrin to the budding IRVs [23]. Thus, formation of the IRVs may involve coordinate action of GGA and ACAP1. In addition, several earlier reports suggest that AP1 and AP3 adaptors may also participate [2,3]. The interplay between different adaptors in the process of IRV formation deserves further investigation.

Both GGA and ACAP1 can interact with Arf6 [23,28], which controls cell surface recycling and regulated secretion in various cell types [29]. In 3T3-L1 adipocytes, the intracellular localization of Arf6 very significantly overlaps with that of GLUT4 on perinuclear donor membranes. More important, depletion of Arf6 in 3T3-L1 adipocytes totally inhibits insulin-stimulated translocation of GLUT4 [23].

The identity of the phosphatidylinositol phosphate that is involved in IRV formation is not yet known. Recently, phosphatidylinositol 4-phosphate (PI4P) was shown to interact with GGA adaptors and to recruit them to the TGN [30,31]. Since the TGN is likely to represent a donor compartment for the GGA-mediated IRV formation, PI4P emerges as a plausible candidate. The IRVs *per se* are depleted of phosphatidylinositol 4-kinase activity; however, cellugyrin-positive transport vesicles contain PI4K type II α [32]. As these vesicles may deliver IRV component proteins from sorting endosomes to the perinuclear donor membranes, PI4P generation may balance the incoming protein flow with the rate of IRV formation and maintain the integrity of the donor compartment.

Targeting of the newly synthesized IRV proteins

Newly synthesized GLUT4 and IRAP are not targeted to the plasma membrane like most membrane proteins, but arrive in the insulin-responsive compartment within 6 to 9 hrs [26,33,34]. Presumably, these proteins traffic directly from the secretory pathway to IRVs. Reaching the insulin-responsive compartment requires specific targeting signals in the cytoplasmic regions of the proteins. For GLUT4, these signals reside in the N-terminus and large central loop [35]; for IRAP, the dileucine motif at positions 76 and 77 is required [33]. These signals are different from those that navigate the intracellular traffic of recycling proteins [36]. Still, targeting of *de novo* synthesized GLUT4 and IRAP to the insulin-responsive compartment also requires GGA adaptors [26,33]. It remains to be seen whether or not *de novo* synthesized GLUT4 and IRAP mix with recycling proteins in the IRV donor membranes or whether they are targeted to the same insulin-responsive compartment from a different origin.

Trapping and intracellular retention of IRVs

Once formed, IRVs are retained very efficiently within cells not exposed to insulin. Exactly how these vesicles are retained intracellularly is not known, but they likely participate in an intracellular cycle involving endosomes and/or the TGN [2,17,37]. Most recent data suggest that IRVs do not coalesce with transferrin receptor -containing endosomes [37,38]. Moreover, efficient intracellular retention of endocytosed GLUT4 requires SNARE components that drive membrane fusion at the TGN [reviewed in 4]. These components include syntaxin-16, syntaxin-6, Vti1a, and VAMP4 (or VAMP3) in mouse cells. In humans, the complex may consist of syntaxin-16, syntaxin-10, Vti1a, and VAMP3 [12,39]. One possibility is that IRVs recycle *via* the TGN, which could sequester them away from endosomes as well as from the plasma membrane. Such a cycle of budding and fusion accounts for both the enrichment of IRV components and the intracellular retention of these components in unstimulated cells.

It has been proposed that TUG is an essential component of the mechanism responsible for trapping IRVs within cells [4,40,41]. TUG (Tether containing a UBX domain, for GLUT4) was identified in a functional screen for regulators of GLUT4 targeting. A cytosolic protein, TUG binds directly and specifically to GLUT4 and not GLUT1. In 3T3-L1 adipocytes, TUG colocalizes with nonendosomal GLUT4. The TUG-GLUT4 interaction is mediated in part by the large intracellular loop of GLUT4 [41]. Data suggest a second interaction [40], which may involve the GLUT4 N-terminus. As noted above, these regions of GLUT4 are required for insulin-responsive trafficking [35]. The specificity of the TUG-GLUT4 interaction is one piece of evidence supporting the idea that TUG regulates GLUT4 in IRVs, and not at some other location.

The N-terminal and central regions of TUG interact with GLUT4; the TUG C-terminus is not required for this interaction but is essential to retain GLUT4 intracellularly. It was hypothesized that the TUG C-terminus interacts with an “anchoring” protein(s), which may constrain an intracellular cycle required for GLUT4 intracellular retention [40]. Candidate proteins at the TGN have now been identified that may fulfill this role (J.S. Bogan *et al.*, unpublished). In addition, a C-terminal fragment of TUG, UBX-Cter, acts in a dominant negative manner to disrupt intracellular retention of GLUT4 in unstimulated cells [41]. Presumably UBX-Cter acts by binding the “anchoring” site, and preventing the interaction of endogenous, intact TUG at this site. In support of this model, effects of RNAi-mediated TUG depletion and of UBX-Cter expression are indistinguishable: both redistribute GLUT4 to the plasma membrane. Remarkably, the magnitude of this effect is similar to that of insulin action, and there is no large effect on transferrin receptor distribution [40,41]. In cells

containing dominant negative TUG UBX-Cter, GLUT4 that is not at the plasma membrane apparently resides in endosomes. By contrast, TUG overexpression causes GLUT4 to accumulate in nonendosomal vesicles, which are likely IRVs. These findings are consistent with the notion that TUG regulates IRVs and that insulin action at this site is a major point of control.

If IRVs are indeed retained by cycling at the TGN, then IRV components may enter this cycle either directly from the secretory pathway or by retrograde trafficking via endosomes. In either case, the recruitment of TUG to GLUT4-containing membranes is required to trap it, possibly by facilitating fusion at the TGN. The sequential actions of sortilin and TUG may then confer specificity for proteins sequestered in IRVs, and provide a mechanism for sequestration. Because sortilin expression is upregulated during adipocyte differentiation, this model also accounts for the tissue-specificity of IRV regulation [14].

IRV mobilization by insulin

Insulin stimulates dissociation of GLUT4 from TUG, both in 3T3-L1 adipocytes and in muscle [40,42]. Dissociation occurs rapidly, and precedes GLUT4 translocation in 3T3-L1 adipocytes [40,43]. More important, the number of TUG-GLUT4 complexes that dissociate controls the number of GLUT4 proteins that are translocated rapidly upon insulin addition, and that appear as an initial burst at the cell surface. This combination of kinetic and biochemical data initially suggested that TUG controls an IRV pool, which enlarged in cells overexpressing TUG and ablated in cells containing the UBX-Cter dominant negative fragment. These findings indicate a model in which insulin triggers disassembly of the TUG-GLUT4 complex to mobilize IRVs.

TUG contains a UBX domain as well as an N-terminal ubiquitin-like region [40,44]. UBX domains are well known to bind p97/VCP/cdc48, an ATPase that has diverse cellular roles [45,46]. Some cofactors (e.g. p47 and p37) recruit p97 activity to regulate homotypic membrane fusion. Other cofactors (e.g. the Ufd1-Npl4 complex) recruit p97 activity to ubiquitin-dependent protein degradation pathways, including endoplasmic reticulum associated degradation. In both of these examples, p97 is thought to promote the disassembly of protein complexes, and it can target a component of the complex to the proteasome for degradation. Recent work finds that a small fraction of TUG undergoes site-specific endoproteolytic cleavage, and that cleavage is required for insulin to mobilize IRVs (J.S. Bogan *et al.*, unpublished). p97 may participate in this process by extracting a cleavage product and targeting it for proteasomal degradation. Regardless of the precise mechanism, it is attractive to consider the possibility that p97 may facilitate the insulin-stimulated disassembly of a TUG-GLUT4 complex to release GLUT4.

Other proteins that function in ubiquitin-like modification have been implicated in IRV regulation. Ubc9, a SUMO conjugating enzyme, binds GLUT4 and likely controls its trafficking through IRVs [47,48]. Effects of RNAi-mediated Ubc9 depletion were rescued by a catalytically inactive mutant, as well as by the wildtype protein [47]. Thus it remains uncertain if SUMO modification is involved in IRV regulation, and it may be that Ubc9 functions merely as a scaffold. Daxx is another SUMO-binding protein and sumoylation target, which binds a C-terminal sequence of GLUT4 that is required for its accumulation in IRVs [37,49]. However, no clear functional role of Daxx in GLUT4 trafficking has been reported. Whether Ubc9 and Daxx cooperate with TUG and p97 remains to be seen.

The idea that insulin liberates IRVs by disassembling a protein complex fits well with analyses of GLUT4 mobilization. Insulin is proposed to recruit IRVs to the cell surface recycling pathway by a “quantal release” mechanism, in which higher concentrations of insulin liberate greater fractions of the sequestered GLUT4 [37]. The IRVs are apparently

mobilized at random [50], though it is not clear what step is limiting when insulin concentrations are sub-maximal. Distinct insulin signaling pathways may be responsible for IRV mobilization and for fusion at the plasma membrane, and some data suggest that IRV mobilization is independent of Akt and phosphatidylinositol-3-kinase [51–53]. This idea fits with work showing that in “insulin resistant” states, reduced GLUT4 translocation can occur without any reduction in signaling through Akt to AS160/TBC1D4 [54,55]. Possibly, signaling through TC10 is required to mobilize IRVs [7], and Akt activation is required at a later stage in translocation [56].

Finally, it is not known if endocytosed GLUT4 follows the same itinerary in the absence and steady-state presence of insulin. Certainly, it must be targeted to IRVs for sequestration in unstimulated cells, and these IRVs are mobilized upon initial stimulation by insulin. Yet, insulin modulates the endocytic mechanism [57], dephosphorylates syntaxin-16 [58], and alters the activity of particular Rab proteins (e.g. by acting through AS160/TBC1D4) [5]. Therefore, it may be that GLUT4 that is endocytosed during the sustained presence of insulin bypasses the IRV compartment entirely, and returns directly to the plasma membrane from endosomes. Further work is needed to clarify this point.

Conclusions

This summary highlights selected aspects of insulin-regulated GLUT4 trafficking. Much important work on insulin signaling, modulation of Rab activity, and vesicle fusion at the plasma membrane is not covered. It will be interesting to learn which aspects of GLUT4 trafficking are compromised in the setting of insulin resistance, and whether these processes may contribute to the pathogenesis of type 2 diabetes. Additionally, the mechanisms that are involved in IRV cargo recruitment, biogenesis, retention, and release may be adapted to control various other membrane proteins in other tissues [1]. Thus, future work may draw parallels among distinct physiologic processes that share common molecular mechanisms.

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Figure 1. Intracellular GLUT4 trafficking

In unstimulated cells, GLUT4 is localized predominantly in the perinuclear region in small, insulin-responsive vesicles (IRVs). In addition to GLUT4, these vesicles contain IRAP, LRP1, and VAMP2, as well as sortilin, a cargo adaptor protein. The IRVs exist in a dynamic equilibrium with donor membranes that are likely a sub-domain of the trans-Golgi network (TGN) and/or recycling endosomes. Formation of the IRVs requires GGA and ACAP1 adaptor proteins, clathrin, and probably phosphatidylinositol 4-phosphate (PI4P). Once formed, IRVs are retained intracellularly by TUG, Ubc9, and other proteins, which may constrain an intracellular cycle of budding and fusion. Upon insulin addition, IRVs are mobilized and fuse with the plasma membrane. The IRV component proteins are internalized into sorting endosomes and returned to donor membranes by cellugyrin-containing transport vesicles. As diagrammed, the IRVs are labeled in magenta, proteins and lipids are denoted in blue, donor membranes in red, and retention and mobilization steps are in green.