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REVIEW

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GLUT4 Storage Vesicles: Specialized Organelles for Regulated Trafficking

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Fat and muscle cells contain a specialized, intracellular organelle known as the GLUT4 storage vesicle (GSV†). Insulin stimulation mobilizes GSVs, so that these vesicles fuse at the cell surface and insert GLUT4 glucose transporters into the plasma membrane. This example is likely one instance of a broader paradigm for regulated, non-secretory exocytosis, in which intracellular vesicles are translocated in response to diverse extracellular stimuli. GSVs have been studied extensively, yet these vesicles remain enigmatic. Data support the view that in unstimulated cells, GSVs are present as a pool of preformed small vesicles, which are distinct from endosomes and other membrane-bound organelles. In adipocytes, GSVs contain specific cargoes including GLUT4, IRAP, LRP1, and sortilin. They are formed by membrane budding, involving sortilin and probably CHC22 clathrin in humans, but the donor compartment from which these vesicles form remains uncertain. In unstimulated cells, GSVs are trapped by TUG proteins near the endoplasmic reticulum – Golgi intermediate compartment (ERGIC). Insulin signals through two main pathways to mobilize these vesicles. Signaling by the Akt kinase modulates Rab GTPases to target the GSVs to the cell surface. Signaling by the Rho-family GTPase TC10α stimulates Usp25m-mediated TUG cleavage to liberate the vesicles from the Golgi. Cleavage produces a ubiquitin-like protein modifier, TUGUL, that links the GSVs to KIF5B kinesin motors to promote their movement to the cell surface. In obesity, attenuation of these processes results in insulin resistance and contributes to type 2 diabetes and may simultaneously contribute to hypertension and dyslipidemia in the metabolic syndrome.

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†Abbreviations: ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartments; ACAP1, ADP-ribosylation factor (ARF) GAP with coiled-coil, ANK repeat and pleckstrin homology domains; GSV, GLUT4 storage vesicles; IRAP, insulin-regulated aminopeptidase; LRP1, lipoprotein receptor-related protein 1; PDK, phosphatidylinositol-dependent kinase; PIP3, phosphatidylinositol 3,4,5-triphosphate.

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INTRODUCTION

The trafficking relationships among membrane-bound organelles involved in secretory physiology were famously described by Jamieson and Palade in their pioneering work on pancreatic exocrine cells [1]. These and subsequent studies established the canonical endoplasmic reticulum (ER) to Golgi to secretory vesicle pathway that is used in diverse cell types. Since then, additional membrane-bound compartments have been identified, and are often derived from the ER/Golgi pathway, including peroxisomes, lipid droplets, omegasomes and autophagosomes, the ER-Golgi intermediate compartment (ERGIC), and others [2]. It is now recognized that membrane trafficking has functions in addition to regulated secretion, such as non-secretory exocytosis to deliver transmembrane proteins to the cell surface action, as well as exocytic processes that expand the plasma membrane [3].

In this review, prepared for a focus issue of *Yale Journal of Biology and Medicine* on Organelles, we describe on how work investigating the trafficking dynamics of GLUT4, the major insulin-responsive glucose transporter, revealed the existence of a novel class of intracellular organelle [4]. Specifically, data support the concept that in fat and muscle cells, GLUT4 is retained in small 50-70 nanometer vesicles where it is co-localized with other proteins that have very specific functions involving water and lipid homeostasis. These vesicles bypass the traditional secretory pathway and are sequestered near the ERGIC as a pre-formed pool, where they are primed for insulin-stimulated translocation [4,5]. The mechanisms by which these vesicles are formed and are regulated are still not defined, and it remains uncertain how this membrane-bound organelle buds from donor membranes, how cargoes are selectively incorporated, how the vesicles are retained, and how insulin stimulates the mobilization of these vesicles. The emerging notion that homologous organelles may be present in other cell types represents an interesting new development in the understanding of subcellular organization.

IDENTIFICATION OF RETAINED SMALL VESICLES AS AN INDEPENDENT TRAFFICKING COMPARTMENT

The existence of a novel membrane bound compartment in adipose and muscle cells was anticipated by mathematical modeling of GLUT4 trafficking kinetics. Analyses implied that GLUT4 traffics not just between the plasma membrane and endosomes, but also through an additional, intracellular "insulin-responsive compartment" [6]. The modeling implied that the main trafficking rate affected by insulin is that for exocytosis of GLUT4 from this compartment [7,8]. Remarkably, the escape of GLUT4 from this reservoir in unstimulated cells can be undetectable, so that the main effect of insulin is to expand the pool of GLUT4 that cycles at the plasma membrane [9-12]. Insulin acts on the sequestered GLUT4 to release discrete quanta, in a dose-dependent manner, into a cell-surface recycling pathway [13]. This "static retention" model adapted an earlier "retention receptor" concept [14], based on the new recognition that retention segregates GLUT4 away from endosomes. This static model seemed initially to be incompatible with a "dynamic equilibrium" model [15,16], but subsequent data resolved these discrepancies and showed that insulin also acts dynamically to increase exocytosis from endosomes [11,17,18]. Live cell imaging further supported the idea that, in unstimulated cells, most insulin-responsive GLUT4 is sequestered in a static compartment in cultured 3T3-L1 adipocytes [19] and *in vivo* in muscles in mice [20,21].

The idea that GLUT4 is trapped in a unique, intracellular membrane-bound compartment – called "GLUT4 Storage Vesicles" (GSVs) or "Insulin-Responsive Vesicles" (IRVs) – was also supported by biochemical and electron microscopy (EM) data. Early immuno-EM studies of unstimulated fat and muscle showed that ~75 percent of total intracellular GLUT4 resides in 50-80 nm diameter vesicles and tubules [22]. The remaining GLUT4 is in larger structures, likely endosomes and the trans-Golgi network (TGN). Biochemically, the insulin-responsive GLUT4 can be purified away from endosomes and other organelles [23-25]. Yet, the use of physicochemical characteristics to purify these small vesicles results in the isolation of two distinct pools of vesicles, including GSVs, which are rapidly mobilized by insulin, and intracellular transport vesicles, which are not mobilized and can be distinguished by the presence of cellugyrin [26,27]. The cellugyrin-containing vesicles may provide a reservoir to replenish the GSVs [28]. The GSVs are considered to exist as a pre-formed pool of vesicles within unstimulated cells, based primarily on *in vitro* reconstitution of vesicle budding [29]. This is important, because it implies that the main effect of insulin is not to stimulate a membrane budding process, but rather to act through some other mechanism to mobilize vesicles from this sequestered pool. As discussed further below, effects on membrane fusion also cannot account for release of the GSVs, and insulin-stimulated cleavage of TUG proteins is a mechanism to release GSVs that fits with the data.

Systematic analysis of proteins residing in GSVs reveals that the core constituents of these vesicles are GLUT4, IRAP, sortilin, and LRP1 [24,25,27]. IRAP is an Insulin-Responsive AminoPeptidase, which is thought to co-traffic with GLUT4 throughout most of its intracellu-

lar itinerary. Sortilin plays a role in recruiting cargoes into the GSVs during vesicle budding and binds LRP1 (lipoprotein receptor-related protein 1), as discussed below. In addition, GSVs contain Syntaxin-6 (Stx6) and VAMP2, which is the main v-SNARE required for fusion of these vesicles at the cell surface. Recently, TUSC5/TRARG1 was identified as another protein that participates in GSV trafficking, although precisely how this protein acts remains unknown [30,31]. Several other proteins are also enriched in GSVs [32]. These proteins all translocate to the plasma membrane after insulin stimulation and are dramatically depleted from the intracellular GSV pool. The regulation of these small vesicles therefore represents a unique mechanism by which a set of proteins can be held inactive in a sequestered organelle, and then coordinately mobilized to the cell surface in response to a specific, extracellular stimulus.

FORMATION OF GLUT4 STORAGE VESICLES

One question raised by the discovery of GSVs is how this compartment can be reconstituted after insulin-stimulated depletion. Cargoes must be sorted into GSVs with high fidelity, and the different endocytic rates of various cargoes requires a specific sorting step take place during GSV formation [33]. Studies of GSV formation are complicated by the fact that these vesicles are concentrated in the perinuclear region, so that conventional fluorescence microscopy is not able to distinguish these vesicles unambiguously [34]. Biochemically, the GSVs may participate in an intracellular cycle of budding and fusion with larger, "donor" membranes from which they are formed [15,35]. A further potential complicating factor is that GLUT4 and other cargoes must be able to arrive in this compartment from both biosynthetic and recycling pathways. Newly synthesized GLUT4 and IRAP are not targeted to the plasma membrane, but enter GSVs directly within 6 to 9 hours after they are translated on ER membranes [36-38]. As well, GSV component proteins that have been delivered to the plasma membrane are internalized into sorting endosomes and are delivered to recycling endosomes and/or the trans-Golgi network [25,39,40]. The cargoes, too, must be sorted to a "donor" membrane compartment and into GSVs. It remains uncertain at what point the biosynthetic and endocytic routes reach confluence; regardless, the partitioning mechanism must be able to recognize cargoes arriving from both pathways.

Specific targeting signals have been identified in the cytosolic regions of the main GSV cargoes, which are required for the proteins to reach the GSVs and for these vesicles to be mobilized upon insulin stimulation. For GLUT4, a 12-transmembrane domain protein, these signals reside in the cytosolic N-terminus and large central loop [41]. In particular, the N-terminus contains a critical phenyalanine-based motif, which appears to regulate GLUT4 in GSVs as well as its internalization from the plasma membrane [8,41,42]. In IRAP, which has a cytosolic N-terminus followed by a single transmembrane and a large extracellular domain, a dileucine motif at positions 76 and 77 is required for insulin-regulated trafficking in GSVs [37]. These motifs are distinct from those governing intracellular trafficking of recycling proteins [43]. These signals were predicted to interact with proteins that are peripherally associated with GSVs, and to permit the regulation of GSV trafficking by such proteins [27]. This prediction is fulfilled by TUG, which interacts with these cytosolic regions of both GLUT4 and IRAP [44-46]. Another protein that is recruited by binding IRAP is AS160/Tbc1D4, a GTPase Activating Protein (GAP) for Rab proteins involved in GLUT4 trafficking [47,48]. The IRAP-AS160 interaction may be involved in recruiting IRAP from endosomes into GSVs [49]. The data imply that both AS160 and TUG are present on GSVs, and these proteins likely act together. If GSVs are one example of a more general organelle for regulated exocytic translocation, as we propose, then cytosolic signals similar to those on GLUT4 may be present on other cargoes present in other cell types.

In parallel to this work to define cytosolic signals displayed outside of the GSVs, it was found that GLUT4, IRAP, LRP1, and sortilin interact with each other through sequences present in the vesicle lumen [25,34,35,50]. These luminal interactions may bring cargoes in the donor membranes together, during vesicle budding, so that oligomeric complexes are formed to facilitate protein sorting into the GSVs. The key scaffold for this process is sortilin, a homolog to the yeast vacuolar sorting receptor Vps10p. Sortilin is a multi-ligand protein receptor with a DXXLL motif in its cytosolic domain, through which it recruits clathrin adaptors such as GGA2 to promote vesicle budding [51]. Sortilin thus functions as a transmembrane scaffold to link GSV transmembrane cargoes with peripherally associated adaptors involved in vesicle budding. Its cell type-specific expression in adipocytes is necessary for GSV formation, and exogenous expression of sortilin in preadipocytes is sufficient to drive formation of GSV-like vesicles [35,50,52].

Another adaptor protein that may act on GSVs or on other GLUT4-containing vesicles is ACAP1, which binds to the central loop of GLUT4 [26,53]. ACAP1 has GAP activity toward ARF6, a GTPase involved in vesicle budding, and also recruits clathrin coats. It may work together with sortilin. Ubiquitination is another signal on GLUT4 itself that binds GGA proteins to drive vesicle budding, and which is required for entry of GLUT4 into GSVs [54]. Finally, in human but not mouse cells, muscle- and adipose- specific expression of a clathrin heavy chain,

CHC22, is involved in regulated GLUT4 trafficking and is required for insulin-responsiveness [55-57]. It seems likely that this protein acts in the formation of GSVs themselves, and not at some other site in the intracellular itinerary of GLUT4. Yet, the specific membrane of origin at which this unique clathrin coat acts to promote the budding vesicles remains to be fully elucidated.

FUNCTIONS OF GSV CARGO PROTEINS

The distinct GSV cargo proteins are anticipated to act in membrane trafficking or in organism-level physiology. These can highlight the physiological significance of this organelle and its regulation by insulin signaling.

GLUT4 and Glucose Transport

Postprandial glucose uptake into muscle and adipose tissues is a key homeostatic function for mammals that is regulated by insulin [58]. Insulin promotes hepatic glycogen synthesis, suppresses hepatic gluconeogenesis, increases adipose and muscle glucose uptake, and suppress lipolysis [59]. Together, these functions allow for storage of glycogen and triglyceride, which can serve as energy sources for future mobilization [60].

Glucose uptake from the circulation in humans is mediated by a family of facilitative transporters, known as GLUTs, that are energy-independent and transport glucose across cellular membranes down its concentration gradient [61]. Although several structural components are conserved among all GLUT family members, including 12 transmembrane helices, GLUT isoforms vary in terms of tissue-specific expression and substrate specificity [62,63]. Among the GLUT proteins, GLUT4 is unique because it is expressed primarily in adipose and muscle tissues, and it is sequestered intracellularly in GSVs in cells not stimulated by insulin [22,64]. The regulated, exocytic translocation of GSVs promotes glucose uptake by inserting GLUT4 into the plasma membrane.

Genetic manipulation of GLUT4 in muscle and adipose in mice demonstrated its critical importance for overall glucose homeostasis and revealed crosstalk between tissues [65,66]. During the development of type 2 diabetes, in insulin resistant states, insulin-regulated GLUT4 trafficking is impaired. Although this may in part reflect attenuation of insulin signaling [59], older studies imply that the GSVs are not properly formed and/or retained within unstimulated cells [67,68]. The importance of GLUT4 in glucose uptake and diabetes pathogenesis has been extensively reviewed previously and will not be discussed further here [4,64,69]. Of note, similarly increased glucose uptake was observed after insulin stimulation and TUG disruption, both in 3T3-L1 adipocytes and *in vivo* in muscle in mice [44,70]. In both cases, insulin had a minimal further effect in the setting of TUG disruption. These data imply that control of the GSVs by TUG is a major site of insulin action to regulate overall glucose uptake into fat and muscle.

IRAP and Vasopressin Degradation

Like GLUT4, Insulin-regulated aminopeptidase (IRAP) translocates to the plasma membrane in response to insulin [71]. Cloning of the cDNA revealed that IRAP is an aminopeptidase consisting of a cytoplasmic N-terminal tail, a single transmembrane segment, and an intraluminal/extracellular C-terminal domain [72,73]. Though the molecular weight of IRAP is theoretically 120 kDa the protein is known to be heavily glycosylated such that the molecular mass of the processed protein is significantly larger, in the range of 165 kDa [74]. IRAP has a wide tissue distribution, but is most notably expressed in skeletal muscle cells and adipocytes, as well as in the corresponding adipocyte and muscle cell lines, 3T3-L1 adipocytes and L6 myotubes, respectively [75,76]. In these cells, insulin stimulation leads to a six- to eight-fold increase of IRAP at the cell surface within ~5 minutes [45].

Although IRAP's function at the time of its identification was unclear, subsequent studies have shown that it is important in the regulation of water homeostasis via its role in degrading vasopressin. This function was established by experiments demonstrating that vasopressin did not undergo N-terminal proteolysis in the presence of IRAP-deficient muscle or fat cells [77]. This decrease in proteolytic activity corresponded to a two-fold elevation of endogenous vasopressin in the serum of IRAP deficient mice. Although the clearance of vasopressin in IRAP-deficient mice also suggested that additional peptidolytic pathways may exist for the processing of vasopressin, these were unable to compensate for the absence of IRAP-mediated cleavage [77]. Furthermore, insulin stimulation of fat and muscle cells increased the clearance of circulating vasopressin by ~30 percent in wildtype control mice, measured using a ^{125}I-vasopressin label, and this effect was absent in IRAP-deficient mice. Notably, mice lacking IRAP also had a 3-fold increase in the half-life of circulating vasopressin, as well as a compensatory decrease in vasopressin abundance in brain, where vasopressin is produced. Conversely, an increased rate of vasopressin inactivation is observed when IRAP is displayed constitutively at the cell surface. Specifically, in transgenic mice with disrupted TUG-mediated GSV intracellular retention in muscle, IRAP is translocated to T-tubule membranes and results in accelerated inactivation of circulating vasopressin [45]. This caused an impairment in urine concentration by the kidneys, so that the mice consumed more water, compared to wildtype control animals. Together, the data support the concept that the regulation of IRAP at the cell surface has marked

effects on vasopressin turnover.

LRP1 and Wnt Signaling

The low-density lipoprotein receptor-related protein 1 (LRP1 or CD91) is a transmembrane protein belonging to the low-density lipoprotein receptor (LDL-R) family [78]. LRP1 binds and internalizes more than 40 unrelated ligands, such as the α2-macroglobulin-protease complex (α2M) and triglyceride-rich lipoproteins bearing apolipoprotein E (apoE) [79]. In addition, the endocytosis and intracellular trafficking of LRP1 plays a key role in regulating the cellular functions and activities of other receptors and plasma membrane proteins that interact with LRP1, such as platelet-derived growth factor receptor β, insulin receptor (IR), and insulin-like growth factor receptor-1 (IGFR-1) [80]. Finally, LRP1 modulates Wnt signaling, which also impacts cholesterol and fatty acid metabolism [81-83].

More specifically with regards to its role within muscle and fat tissue, LRP1 has multiple functions in regulating cholesterol and lipid metabolism. For example, LRP1 participates in ApoE-mediated uptake of triglyceride-rich lipoprotein remnants such as chylomicrons and very low-density lipoproteins [84]. Insulin-induced LRP1 translocation correlates with increased postprandial chylomicron remnant uptake [85]. LRP1 also binds another apolipoprotein, ApoA-V, which is present both on lipoprotein particles and also on intracellular lipid droplets; LRP1 thus acts to control adipocyte triglyceride metabolism [86]. ApoA-V treatment of cultured adipocytes leads to decreased triglyceride uptake and may also contribute to increased lipolysis. Thus, the data suggest that regulation of LRP1 within GSVs may have an important effect to control lipid homeostasis, and to coordinate this with glucose uptake.

VAMP and Membrane Fusion

Vesicle associated proteins (VAMPs) are the vesicle (v-)SNARE proteins that mediate membrane fusion. VAMP2 and VAMP3 are concentrated in GSVs, and their cleavage by botulinum neurotoxin D caused impaired GLUT4 translocation [87,88]. These two VAMP isoforms may be present in different GLUT4 pools, as VAMP3 containing GLUT4 vesicles had almost unnoticeable levels of VAMP2 [87]. VAMP3 is important in constitutively recycling pathways and reduction of the protein does not interfere with insulin dependent translocation of VAMP2 containing GLUT4 vesicles [89]. In muscle, VAMP3 is limited to endosomal pathways, while VAMP2 is similarly present within insulin-responsive GLUT-4 vesicles [90].

Studies with VAMP3 null mice found that its function is not necessary for normal glucose metabolism, GLUT4 translocation, and constitutive membrane recycling [91]. Furthermore, syntaxin-4 and SNAP23 form ternary complexes, together with VAMP2 and the Sec1-Munc18 (SM) protein Munc18c. This SNARE-SM complex is thought to play an integral role in fusion of GSVs at the plasma membrane [92,93]. VAMP2 has broader functions as well and plays a role in the release of hormones from pancreatic islet cells, neurons, and gastric parietal cells [94,95].

INSULIN SIGNALING, GLUT4 TRAFFICKING, AND GSV REGULATION

The regulation of GLUT4 translocation to the plasma membrane requires the intersection of insulin signaling and vesicle trafficking pathways. Yet, many of the vesicle trafficking processes that are involved in GLUT4 targeting are not major sites of insulin regulation. A key challenge for the field has been to understand not only what steps are regulated by insulin, but what step is quantitatively most important for the control of glucose uptake. As noted, mobilization of a preformed pool of GSVs is highly regulated, and does not involve membrane budding, sorting, or fusion. We propose that this action is controlled by TUG cleavage, as detailed below. Of course, insulin does regulate classical membrane trafficking processes as well. For example, the fusion of vesicles at the plasma membrane is highly regulated by insulin [96]. Yet, the availability of vesicles for this fusion depends upon an upstream step, in which the GSVs are first mobilized from their intracellular sequestration. The GSVs do not accumulate in a tethered or docked state at the plasma membrane of unstimulated cells but are trapped more deeply within the cell while awaiting an insulin signal. A further consideration is that the major insulin-regulated step may not be the one that is most affected in insulin resistance. Thus, a detailed map of GLUT4's intracellular itinerary, and of the sites that are regulated by insulin and affected in insulin resistance, is necessary to understand physiology and pathophysiology.

In unstimulated cells, GLUT4 and other GSV cargoes are internalized by endocytosis into early (or sorting) endosomes [97]. These cargoes then enter recycling endosomes and follow a retrograde pathway to the trans-Golgi network and possibly to the ERGIC or other donor membrane compartments [4]. GSVs then bud from these membranes and are held in a relatively static configuration to await insulin stimulation. An insulin signal then releases the trapped GSVs and loads these vesicles onto microtubule motors for translocation to the plasma membrane (Figure 1). As detailed below, intact TUG can trap the GSVs, and TUG cleavage can liberate these vesicles and load them onto microtubule motors. After translocation, GSV cargoes recycle through endosomes during ongoing insulin exposure [4,17]. GSV vesicle budding,

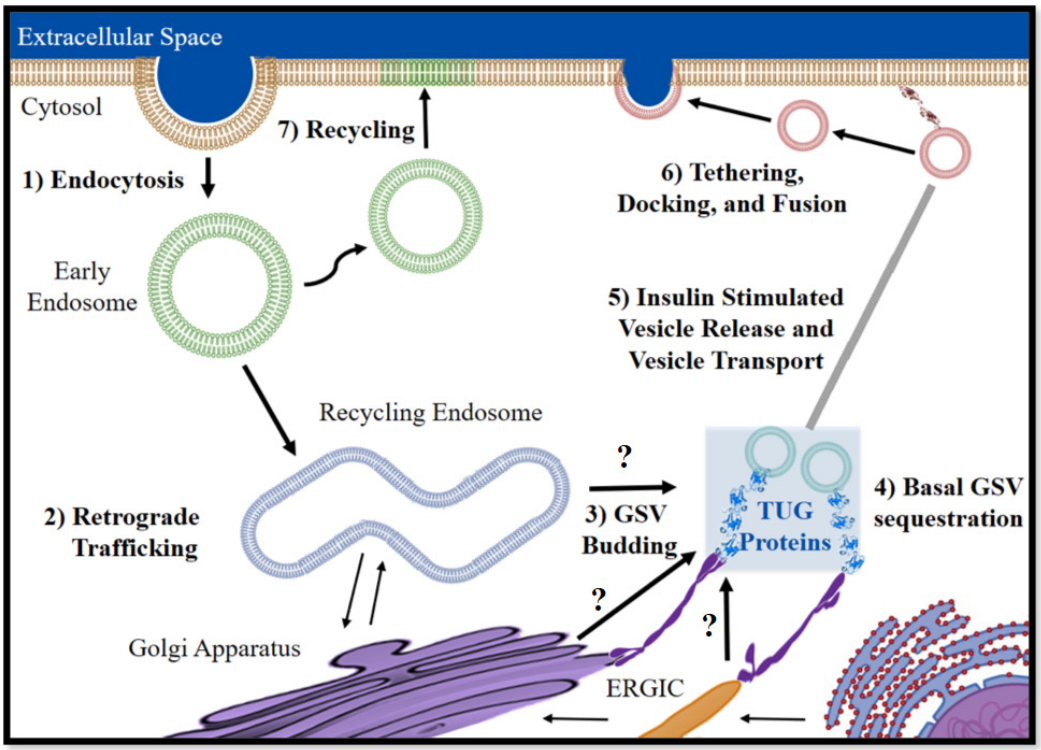

Figure 1. GLUT4 trafficking pathways. After GLUT4 undergoes endocytosis from the plasma membrane (**1**), it undergoes retrograde trafficking through recycling endosomes to donor membranes such as trans-Golgi network and the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) (**2**). GLUT4 storage vesicles (GSVs) then bud from donor membranes (**3**) and are sequestered by TUG proteins in a static configuration (**4**). Insulin stimulates TUG cleavage subsequent loading of GSVs onto kinesin motors for transport to the cell surface (**5**). GSVs are tethered, docked, and fused with the plasma membrane (**6**) to insert GLUT4. During ongoing stimulation by insulin, GSV components may return to the plasma membrane directly from endosomes, thereby bypassing the GSV compartment. The origin of GSVs remain largely unknown or not fully described.

retention, insulin-stimulated release, cytoskeletal transport, and vesicle targeting must all be highly coordinated.

Disruption of TUG-mediated GSV retention results in increased lysosomal degradation of GLUT4, as well as in its targeting to the plasma membrane, in cultured 3T3-L1 adipocytes [44]. It is not clear whether this results from lysosomal targeting of GLUT4 present in endosomes, or whether the GSVs themselves may fuse with a lysosomal compartment when TUG-mediated retention is disrupted. As noted above, the luminal Vps10p domain of sortilin binds the first luminal loop of Glut4. This may prevent endocytosed GLUT4 from entering lysosomes, because the cytoplasmic tail of sortilin then binds retromer to facilitate GLUT4 retrograde trafficking [98]. Of note, inhibition of retromer function decreases the stability of GLUT4 and blocks entry of endocytosed GLUT4 into the insulin-responsive GSV compartment. Some data support the idea that retromer subunits are translocated to the plasma membrane after insulin stimulation, yet knockdown of the retromer protein, VPS35, as well as Sorting nexin-27 (SNX27), led to confounded effects including impaired adipocyte differentiation, as well as impaired GSV biogenesis [99]. Retrograde trafficking of GSV cargoes is eventually coupled to their capture by golgins, particularly Golgin-160 and p115, which engage vesicles at the cis-side of the Golgi complex [100-102].

Broadly, insulin signaling pathways that coordinate GLUT4 regulation can be divided into those centered on the serine/threonine kinase Akt [103,104], the cytoskeletal factors such as Rho-family small GTPase Rac1, and wortmannin insensitive pathways including that involved in vesicle fusion at the plasma membrane [105] and that involving TC10α, PIST, and TUG [106,107]. Both the Akt and TC10α–PIST–TUG pathways impinge on GSVs, and the Akt pathway likely also directs GLUT4 in endosomes to return to the plasma membrane during ongoing insulin exposure. The Rac1 pathway regulates actin remodeling at the cell cortex and facilitates GLUT4 translocation during muscle contraction as well as after insulin stimulation [108,109]. Vesicle fusion at the plasma membrane involves a VAMP2-SNAP23-Syntaxin-4 SNARE complex, and is regulated by insulin-stimulated

phosphorylation of the SNARE regulator Munc18c as well as by action of Munc13 and DOC2B [110].

Insulin action through the classical Akt pathway involves tyrosine phosphorylation of insulin receptor substrate proteins (*e.g.* IRS1), which recruit phosphatidylinositol-3-kinases (PI3K) to generate phosphatidylinositol 3,4,5-triphosphate at the inner leaflet of the plasma membrane. This recruits Akt, which is phosphorylated on Ser473 by mTORC2 and on Thr308 (Thr309 in Akt2) by phosphoinositide-dependent protein kinase 1 (PDPK1) [4,111]. The second of these is the main site required for GSV translocation, and activates Akt activity to phosphorylate AS160/Tbc1D4, a Rab GTPase Activating Protein (GAP) that controls vesicle trafficking. As noted above, AS160 binds IRAP and is likely present on GSVs [47,48]. Insulin action through both AS160/Tbc1D4 and the related Tbc1D1 protein has recently been reviewed [32,112]. Phosphorylation inactivates the GAP activity of these proteins, and so activates downstream Rab proteins. In particular, this is thought to activate Rab10 on GSVs in adipocytes and Rab8A, which may act similarly, in muscle cells [32,113].

Data support the idea that GSVs fuse directly with the plasma membrane, and that these vesicles carry Rab10 [17, 18]. Previous literature showed that insulin stimulates the release of GLUT4 from storage compartments into the endosomal recycling system, but this does not imply that the GSVs fuse directly with recycling endosomes [10]. The more recent data indicate that entry of released GSV cargoes into the endosomal system likely follows indirectly, from endocytosis at the plasma membrane, rather than as a direct result of GSV fusion [17,18]. A related question is where the effectors for activated Rab10 or Rab8A are localized, since that may identify a target membrane with which GSVs fuse. A downstream target of Rab10 is Sec16A [114]. Sec16 proteins are described to act as scaffolds for the budding of COPII vesicles at ER exit sites, however the function of Sec16A in GLUT4 trafficking appears to be independent of this function. Its localization is not well defined, and it is not clear if it identifies a target membrane for GSV fusion. Another Rab10 effector is the myristoylated alanine-rich C-kinase substrate (MARCKS), which is present on the plasma membrane [115]. This pathway mediates the insertion of membrane at the cell surface to promote axon development, but it is not clear if MARCKS functions in GSV trafficking. Finally, Rab10 has been suggested to play a role in fusion of lysosomes with the plasma membrane [116], and Rab8A is thought to function through myosin-Va in muscle cells to promote GLUT4 vesicle exocytosis [117]. Further studies will be required to understand how these mechanisms may function together in GSV regulation.

Insulin also signals through the Rho family GTPase TC10α to various downstream targets to translocate GLUT4 [106,118]. Upstream mediators in this signaling pathway may include both positive and negative regulators, so that a feed-forward circuit may be formed and TC10α may be activated transiently, in response to fold-changes in insulin concentration [5,119]. Some of these mediators include CAP and Cbl, which in conjunction with TC10α contribute to actin remodeling dynamics in adipocytes [120]. In fact, TC10α has been shown to regulate both perinuclear and cortical actin, and it binds directly to COPI coat proteins through a dilysine motif to control actin polymerization on membrane transport vesicles [121]. TC10α signaling is coupled to TUG through its effector, PIST, which binds TUG directly and regulates its cleavage [70,102].

TUG AND INTRACELLULAR SEQUESTRATION OF GSVs

The TUG protein, encoded by the *ASPSCR1* gene, was identified in a functional screen for proteins that modulate GLUT4 distribution [46]. Initial data showed that a truncated form of TUG caused a decrease in the size of the insulin-responsive pool of GLUT4; conversely, overexpression of full-length TUG increased the size of the insulin-responsive pool in 3T3-L1 adipocytes. TUG binds specifically to GLUT4, but not GLUT1, and regulates the trafficking of GLUT4 but not endosomal proteins. Insulin stimulates the dissociation of intact TUG from GLUT4; this precedes GLUT4 translocation and the number of TUG-GLUT4 complexes that are dissociated controls the magnitude of the initial translocation response. Based on these data, it was hypothesized that TUG binds an intracellular anchoring site and traps the GSVs at this location, and that insulin then releases this tether to mobilize the GSVs. TUG was named as a functional "Tether, containing a ubiquitin like UBX domain, for GLUT4."

Subsequent data confirmed and extended this model. RNAi-knockdown studies showed that TUG depletion mimics the effect of insulin stimulation, not only on GLUT4 translocation but on glucose uptake [44]. TUG depletion, like insulin stimulation, reduced GLUT4 protein stability; together these data implied that it acts at a major insulin-regulated step for GLUT4 translocation [44,122]. Other data used total internal reflection fluorescence microscopy (TIRFM) to characterize the rate of vesicle exocytosis in basal and insulin-stimulated, control and TUG-depleted 3T3-L1 cells [17]. Similar increases in exocytosis rates were observed after TUG depletion and insulin stimulation; insulin had only a transient effect in the TUG-depleted cells. Importantly, the approach could distinguish GSVs from endosomes, based on vesicle size, and TUG regulated the exocytosis of GSVs. The data

also showed that immediately after insulin addition, exocytosis of GSVs was increased, but after more prolonged insulin exposure, GSV cargoes recycled to the plasma membrane from endosomes. Thus, insulin causes a switch in the exocytic circuit, possibly by acting through AS160 on Rab14 [18]. Finally, data showed that TUG is localized at the ERGIC. Together with evidence that the GSVs fuse directly at the plasma membrane [17], this suggested that these vesicles follow an unconventional, Golgi-bypass pathway for exocytic translocation [4].

A critical insight to understand how TUG regulates GLUT4 was the demonstration that TUG is cleaved in a site-specific manner at the peptide bond connecting residues 164 and 165, out of 550 total residues in the intact protein [102]. This reaction creates an 18 kDa N-terminal cleavage product and a 42 kDa C-terminal cleavage product. Cleavage separates an N-terminal region of TUG that binds GLUT4 from a C-terminal region that binds Golgi/ERGIC-associated proteins, which shows how cleavage can release GSVs that are trapped at the Golgi/ERGIC. The production of cleavage products was dramatically increased after insulin stimulation [5,102]. Importantly, a cleavage-resistant form of TUG was unable to rescue highly insulin-responsive GLUT4 translocation and glucose uptake in TUG-depleted 3T3-L1 adipocytes [4]. Together, the data showed that TUG cleavage is a critical mechanism by which insulin regulates GSV translocation, and that understanding this biology would be important for elucidating these organelles and their physiologic role.

TUG cleavage is controlled by insulin signaling through TC10α and its effector PIST, which binds directly to TUG [5,102,106,107]. RNAi-mediated depletion of TC10α blocked insulin-stimulated GLUT4 translocation and glucose uptake [106], as well as the production of TUG cleavage products [102]. Remarkably, an unstable fragment of TUG ("UBX-Cter," residues 377-550) recruits PIST for degradation and results in constitutive cleavage of intact TUG in unstimulated cells [70]. These results indicate that PIST normally inhibits TUG cleavage; when loaded with GTP, after insulin stimulation, TC10α removes this inhibition to promote TUG cleavage and GSV mobilization.

The ability of TUG to trap GSVs in an insulin-responsive configuration near Golgi/ERGIC compartments is regulated by acetylation of TUG on lysine residues near its C-terminus [123]. Acetylation modulates the interaction of TUG with Golgin-160 (a cis-Golgi matrix protein) and ACBD3 (acyl-CoA binding domain containing 3), which maintain TUG-bound GSVs in a primed pool ready for insulin-stimulated translocation [123]. Mutation of the acetylated residues caused impairment of GLUT trafficking and blocked TUG cleavage in 3T3-L1 adipocytes, suggesting that these residues are critical for the assembly of a protein complex in which TUG is able to be cleaved. In addition, the NAD^+-dependent deacetylase SIRT2 binds and selectively deacetylates TUG and modulates insulin-stimulated TUG cleavage and glucose clearance *in vivo*. It remains uncertain whether the GSVs are maintained in an entirely static configuration, or if they cycle into and out of Golgi/ERGIC membranes. As well, some data suggest that TUG may be modified by fatty acylation, as well as by acetylation (unpublished); this can also be controlled by SIRT2 [124], and it may link the TUG C-terminus to particular Golgi/ERGIC membrane domains. Action of SIRT2 may shed light how cytosolic reduction-oxidation status may be linked to insulin action and control insulin sensitivity.

TUGUL AND TRANSLOCATION OF GSVs

How is cleavage of TUG linked to translocation of GSVs to the plasma membrane? Early work had shown that GLUT4 translocation requires microtubules and suggested that active transport of GSVs on microtubules by kinesin motor proteins may be involved in this process [125]. These results fit with the idea that GSVs are located in a perinuclear region, some distance away from the plasma membrane. An intriguing hypothesis was that TUG cleavage might both liberate the GSVs from their site of sequestration and also activate microtubule-based transport machinery to carry these vesicles toward the cell surface.

The 18 kDa N-terminal TUG cleavage product contains tandem ubiquitin-like domains and ends in a diglycine motif (residues 163-164), which is typical of ubiquitin-like protein modifiers [102]. Such proteins are covalently attached to target substrates; data showed that the TUG N-terminal cleavage product functions in this manner and it was named TUGUL, for "TUG Ubiquitin-Like." TUGUL modifies ubiquitin itself in transfected cells, but this is likely the result of overexpression of mature TUGUL and not the physiologic substrate. In cultured 3T3-L1 adipocytes, an antibody to the TUG N-terminus detects not only intact, 60 kDa TUG, but also an additional ~130 kDa protein. If TUGUL contributes 18 kDa of this mass, then the physiologic target substrate is predicted to have a relative mass of ~110 kDa. Of note, this 130 kDa TUGUL-modified ("tugulated") protein cofractionates with GSVs and the plasma membrane, implying that it may have a role in GSV trafficking.

Recent data show that the 110 kDa kinesin motor, KIF5B, is the major TUGUL-modified protein in 3T3-L1 adipocytes. KIF5B had previously been shown to carry GLUT4 from the perinuclear region to the cell surface after insulin stimulation [126]. Adipose-specific deletion of KIF5B resulted in glucose intolerance and insulin resistance in mice, supporting the physiological signifi-

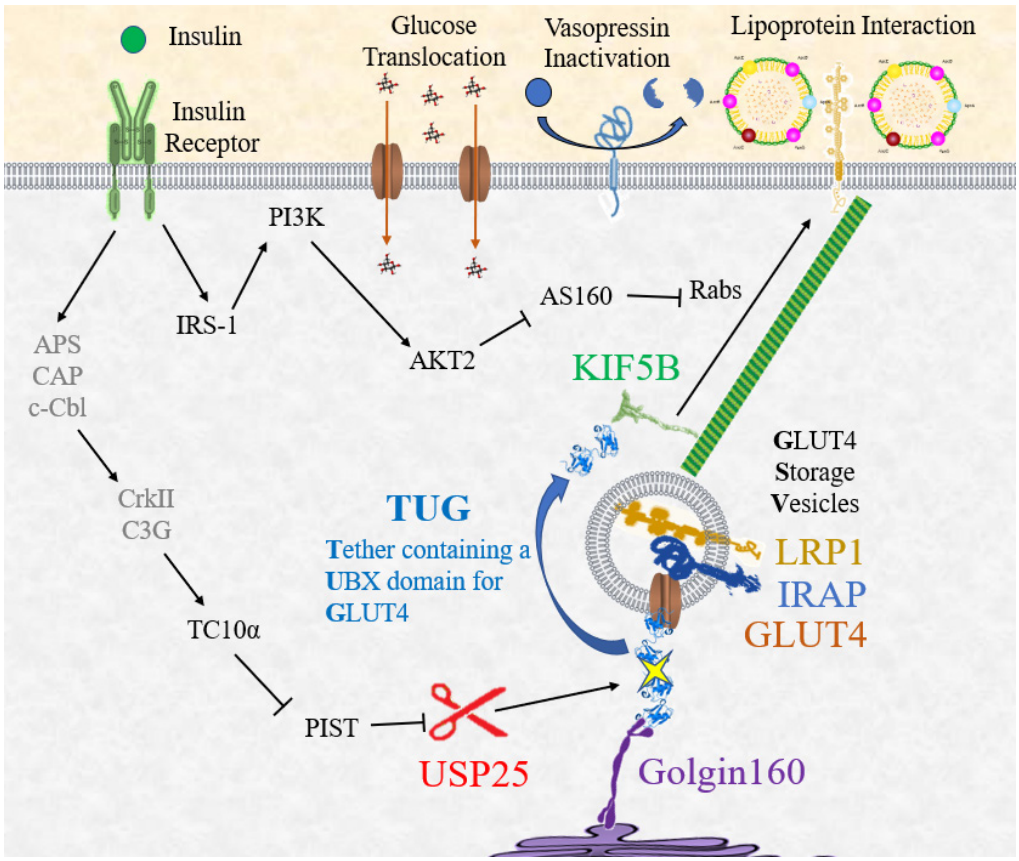

Figure 2. A model for how insulin-stimulated cleavage of TUG and subsequent trafficking of GSVs coordinately regulates multiple physiologic effects. Insulin signals through at least two pathways. Upstream components in the TC10α pathway remain incompletely understood and are shown in gray. Signaling through TC10α removes an inhibitory effect of PIST to trigger Usp25m-mediated TUG proteolysis. Cleavage releases GSVs from an anchoring site comprising Golgin-160 and ACBD3. Acetylation of TUG mediates its binding to this site and controls the size of a GSV pool and, thus, insulin sensitivity. After cleavage, the TUG N-terminal product (TUGUL) modifies the kinesin motor protein KIF5B and translocates with the GSV to the cell surface. The GSV cargos are inserted into the T-tubule membrane in muscle tissue and the plasma membrane in adipose tissue. The GSV cargos mediate glucose uptake (GLUT4), vasopressin inactivation (IRAP), and possibly effects on lipids (LRP1, sortilin).

cance of this protein [127]. In 3T3-L1 adipocytes, insulin activates the KIF5B-dependent movement of GLUT4 in a manner that is insensitive to wortmannin, an inhibitor of insulin signaling through PI3K–Akt [126]. Because TUG cleavage is also stimulated by insulin in a PI3K-independent manner, KIF5B was considered likely to be the main TUGUL-modified protein [128]. Both biochemical and RNAi-mediated depletion experiments confirm this hypothesis, and further show that TUG cleavage is required to load GLUT4 onto KIF5B motors in response to insulin stimulation.

Results further show that a splice form of the Usp25 protease, Usp25m, is expressed in adipose as well as in muscle cells and is the TUG protease [128]. Specifically, Usp25m is both necessary for insulin-stimulated TUG cleavage and translocation of GSV cargoes in cultured 3T3-L1 adipocytes and is also sufficient for TUG cleavage and GLUT4 translocation in transfected cells. Usp25m binds to both TUG and GLUT4 and dissociates from these proteins after insulin stimulation. Together, these studies show that the TUGUL-mediated association of GSVs and KIF5B couples release of the GSVs to activation of transport machinery to carry these vesicles to the cell surface (Figure 2). Precisely how an insulin signal activates Usp25m activity toward TUG is not known, but presumably this process involves activation of TC10α and release of PIST-mediated inhibition. A full understanding of this process will require further work.

PHYSIOLOGICAL IMPLICATIONS OF GSV TRAFFICKING

Table 1. Regulated Non-secretory Exocytic Translocation of Membrane Proteins

Protein	Localization	Function	Signal	Reference
AMPA Receptor	Neurons	Neurotransmission	Calcium / CaMKII	[154]
Aquaporin – 2	Kidney Tubules	Water transport	Vasopressin	[155]
Beta-1-Integrin	Diverse	Cell Migration	Rab1	[156]
CFTR	Lung Epithelia	Chloride transport	Unknown	[159,160]
GLUT4	Adipose / Muscle	Glucose uptake	Insulin	[4,5]
H/K Pump	Stomach	Acidification	Histamine	[95]
H+ Pump	Kidney Tubules	Acid/Base Balance	Vasopressin	[157]
Integrin-alpha	Drosophila	Epithelial Remodeling	dGRASP	[158]

The concept that GSVs serve as a novel organelle that integrates the physiological activities of multiple cargo proteins has significant implications for physiology and pathophysiology. GLUT4 regulation is impaired in states of insulin resistance, which contributes to the pathogenesis of diabetes [129]. Altered GSV trafficking may be causally linked to this pathophysiology in humans, because a mutation found in Greenlandic individuals that causes a premature stop codon in AS160 leads to post-prandial hyperglycemia and increased incidence of diabetes [130]. The more common type 2 diabetes is remarkably prevalent and affects 9 percent of the population in the United States [131]. Diabetes is associated with a wide array of comorbidities including peripheral neuropathy, retinopathy, vasculopathy, and kidney failure and it is now the leading cause of blindness and end stage renal disease in working age adults [129]. Diabetes is also often associated with hypertension, dyslipidemia, and obesity in a constellation known as the metabolic syndrome. Investigation of the pathogenesis underlying insulin resistance in diabetes is one of the reasons that GSVs were discovered.

The colocalization of multiple additional proteins within GSVs, however, suggests that defective GSV translocation may have additional physiologic effects beyond glucose intolerance.

For example, since TUG cleavage integrates translocation of both GLUT4 and IRAP, dysregulation of GSVs may account for the association between insulin resistance and hypertension within the metabolic syndrome [59,132]. Furthermore, since GSVs also contain LRP1, which binds to apolipoproteins such as ApoE and ApoA-V; thus, it is possible that dysregulation of GSVs also contributes to altered lipid metabolism [45]. Understanding these interactions may help to explain otherwise mysterious associations, such as why the vasopressin byproduct copeptin is correlated with insulin resistance in muscle in type 2 diabetes, obesity, and polycystic ovary syndrome [133-143]. Importantly, the impaired regulation of GSV proteins in these resistant states can reflect both increased plasma membrane abundance during the basal state as well as decreased plasma membrane abundance in stimulated states [68]. Thus, the increased copeptin observed in insulin resistant individuals may reflect, at least in part, increased degradation of vasopressin by surface-exposed IRAP. One study demonstrating the potential importance of understanding integrated GSV organelle physiology tested the association between vasopressin pathway single nucleotide polymorphisms (SNPs) and outcomes in patients with septic shock [144]. These investigators found that a particular variant in the IRAP gene was associated with dramatically increased mortality rate, 51.0%, vs 34.5% in control patients, as well as with effects on vasopressin clearance and serum sodium concentrations. Together, the data imply that understanding these coordinately regulated physiologic processes may yield significant translational medical value.

Another example of how regulation of GSVs can have physiological implications is the role of the energy sensor AMPK (5' adenosine monophosphate-activated protein kinase). AMPK is activated in low energy states by sensing the ratio of AMP and ADP to ATP [145]. AMPK is implicated in skeletal muscle glucose transport in response to exercise and can therefore link physical activity to GSV translocation [146]. For example, AMPK is required for insulin-independent GLUT4 translocation during states of energy stress, such as cardiac ischemia or skeletal muscle contraction [147,148]. AMPK activation also enhances the sensitivity of muscle cells to insulin-dependent GLUT4 translocation and glucose uptake and may mediate the enhanced insulin sensitivity that occurs after exercise [149,150]. Finally, recent results show that TUG is cleaved after cardiac ischemia, in an AMPK-dependent manner, which at least partly explains increased GLUT4 and other GSV cargos at the plasma membrane after reperfusion [151]. Together, these effects suggest that understanding how AMPK regulates GSV translocation could be important in diabetes, cardiac ischemia, and other disease states.

CONCLUSIONS AND OUTLOOK

Small Regulated Vesicles in Other Cell Types

It seems likely that GSV-like vesicles are present in other differentiated cell types, where they may regulate the cell surface expression of a wide range of physiologically important membrane proteins. Possibly, this pathway is a cell type-specific adaptation of a more basic cellular pathway. TUG is expressed ubiquitously, although its cleavage is differentiation-dependent. What purpose does intact TUG serve in fibroblasts? Present data imply that it functions in assembly of the Golgi complex, likely in part by controlling the activity of p97/VCP ATPases [152]. Other data show that, in adipocytes, TUG disruption alters the targeting of the SNARE protein, Syntaxin-6 (Stx6) [102]. Stx6 regulates the retrograde trafficking of GLUT4 from endosomes back into GSVs [153]. Yet, Stx6 is also present in a wide range of cell types; thus, we hypothesize that TUG regulates Stx6 function at the Golgi/ERGIC, and this pathway is adapted in a differentiation-dependent manner to control GSVs or GSV-like vesicles. Examples of proteins that may participate in such a pathway for regulated non-secretory translocation have been suggested previously [3], and are also listed in Table 1 [95,154-158]. Not all of these processes need involve TUG cleavage. For example, data suggest that the cystic fibrosis transconductance regulator, CFTR, may participate in a similar trafficking pathway, although not involving TUG cleavage [159,160]. Other potential cargoes in other cell types may also participate in a similar pathway for unconventional, signal-mediated secretion, subject to various upstream regulatory mechanisms. If so, then GSVs may represent the first example of a class of small organelles, in various tissues, that are yet to be characterized and that may have important roles in physiology and disease.

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