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Interactions between the GLUT4 Glucose Transporter and Its Regulator, TUG

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Abstract

The glucose transporter 4 (GLUT4) is the major insulin-responsive glucose transporter in adipose and muscle tissues. Although the early steps in the insulin signaling pathway governing translocation of GLUT4 to the plasma membrane are well understood, the final steps in this pathway are not. TUG is a protein which has been shown to affect trafficking of GLUT4 both in the basal state and in response to insulin. One protein-protein interaction between TUG and the large cytosolic loop of GLUT4 has previously been identified. Based on reports of the requirement of the GLUT4 N-terminal domain for its proper targeting to the plasma membrane, we postulated that an interaction might also exist between TUG and the N-terminal domain of GLUT4, and we tested this hypothesis using two sets of pull-down experiments. In the first set, using the N-terminal domain of GLUT4 fused with glutathione S-transferase (GST), we were able to pull TUG down from the lysates of TUG-transfected HEK 293 cells. TUG was also pulled down by the GLUT4 cytosolic loop and, to a much lesser extent, its C-terminal domain. However, there was no specific interaction between these fusion proteins and the lysates of cells transfected with a truncated form of TUG lacking its own N-terminal domain. In the second set of experiments, using a biotinylated synthetic GLUT4 N-terminal peptide, we pulled down a protein detected by an anti-TUG antibody and running at ~64 kDa, a slightly higher molecular weight than wild-type TUG. We believe that this band represents modified full-length TUG. This interaction was not seen using synthetic GLUT4 N-terminal peptide mutated at 4 amino acids previously identified as necessary for proper GLUT4 retention and insulin-responsive trafficking. We conclude that TUG interacts not only with the large cytosolic loop of GLUT4, but also with the N-terminal domain of GLUT4, and that this latter interaction can be disrupted by mutations in GLUT4 that cause defective trafficking, suggesting that this interaction is critical for GLUT4 intracellular retention and insulin-responsive GLUT4 trafficking.

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Introduction

Type 2 diabetes is a large and growing public health concern worldwide with both microvascular and macrovascular complications, including kidney failure, stroke, and myocardial infarction. The hallmark of type 2 diabetes is elevated plasma glucose secondary to insulin resistance in peripheral tissues. Among its many effects on the body, insulin causes increased glucose uptake in muscle, adipose, and hepatic tissues. This effect is mediated primarily by the facilitative glucose transporter GLUT4. Insulin acts on adipose and skeletal muscle tissue to rapidly redistribute GLUT4 from within the cell to the plasma membrane, where it can then transport glucose from the bloodstream and interstitial space into the cell, thus regulating glucose levels in the blood. Other members of the glucose transporter family, such as GLUT1, exist primarily at the plasma membrane in the basal state, and their intracellular pools do not accumulate as strongly in response to insulin. As discussed below, defects in GLUT4 trafficking and/or expression are now considered to be the common steps in, and main causes of, insulin resistance and type 2 diabetes.

Early discoveries in glucose transport

Starting in the late 1940's and continuing through the 1950's, Rachmiel Levine and colleagues demonstrated that cells in the body were not permeable to blood sugars such as glucose, and that insulin dramatically increased their volume of distribution (1). The "transport theory" proposed that insulin was responsible for stimulating the transport of glucose into tissues, and that this resulted in lower blood glucose levels. It was initially

thought that insulin might activate transporters that were already in the plasma membrane. Because the kinetics of the insulin response were too rapid to be mediated by mRNA transcription or protein translation, insulin's action would require pre-formed transporters. However, in the late 1970's, two groups independently proposed the 'translocation hypothesis', which was an unexpected mechanism at the time (2, 3). This theory was that the glucose transporter was located intracellularly and required the presence of insulin to translocate it to the plasma membrane. It took several more years to identify the first glucose transporter GLUT1 (4). In 1989, different groups identified and cloned the major insulin-responsive glucose transporter GLUT4 (5, 6), which was located primarily in insulin-responsive tissues and did indeed translocate rapidly in reponse to insulin, supporting the translocation hypothesis proposed by Cushman and Kono (2, 3).

The role of GLUT4 in diabetes

Since the initial cloning of GLUT4, there has been significant progress in determining its role in insulin resistance and type 2 diabetes, both through animal models and through studies of endogenous GLUT4 in humans. GLUT4 heterozygous knockout mice developed significant insulin resistance (7). Moreover, mice deficient in GLUT4 selectively in adipose tissue (8) or muscle alone (9) also exhibited considerable insulin resistance, not only in the targeted tissues and the body as a whole, but also in tissues not directly targeted (for review, see (10)). In the adipose-selective GLUT4 knockouts, the reason for insulin resistance in non-adipose tissues is multifactorial, and is in part due to increased secretion of retinol binding protein-4 (RBP4) into serum by adipocytes (11).Thus in addition to causing direct glucose uptake, GLUT4 translocation in

adipocytes might trigger signaling events that regulate the secretion of RBP4 and other adipocytokines which directly modulate insulin sensitivity in other tissues.

Muscle and fat tissue from type 2 diabetics has been found to be deficient in levels of GLUT4 at the plasma membrane, and it is thought that this defect underlies insulin resistance in this population. Overall GLUT4 levels are decreased in adipose tissue but unchanged in muscle. The role that decreased levels of GLUT4 in adipose tissue plays in diabetes is still in question. Nevertheless, in both the adipose and muscle tissue of type 2 diabetics, GLUT4 translocation is defective (12-14). The

GLUT4 trafficking

In order to fully understand GLUT4 trafficking, we must understand it in both the basal state and in response to insulin. Much of our knowledge about GLUT4 trafficking comes from 3T3-L1 cells, a widely used mouse-derived adipocyte model. In the basal state, a relatively low amount of GLUT4 is found at the plasma membrane, with approximately 90% located intracellularly (15).

These low levels of GLUT4 at the plasma membrane are determined in part by the relatively fast rate of endocytosis and slow rate of exocytosis. The GLUT4 found at the plasma membrane in the basal state was thought to largely undergo endocytosis via clathrin-coated pits. A recent study suggests that GLUT4 is actually internalized by two separate mechanisms: ~80% by a nystatin-sensitive, AP-2 clathrin adaptor protein-independent mechanism, and the remainder by a nystatin-insensitive, AP-2-dependent mechanism (16). The authors demonstrate that insulin acts to inhibit primarily the nystatin-sensitive pathway. In 3T3-L1 adipocytes, half of all GLUT4 in the cell

colocalizes with the transferrin receptor (TfR), which cycles between the *trans*-Golgi network (TGN) and plasma membrane via recycling endosomes (RE). The other half is located predominantly in a different pool, termed glucose storage vesicles (GSV's). In real adipocytes, there is significantly more GLUT4 found in GSV's, with relatively little GLUT4 found in endosomes, and this balance might be different in muscle. One model which ties together the various observations about GLUT4 trafficking in the basal state posits that there are two separate cycling pathways (Figure 1). The first cycle is between the plasma membrane and recycling endosomes via early endosomes. The second cycle is between recycling endosomes and the TGN via transport vesicles in the retrograde direction and GSV's in the anterograde direction (for review, see (17)). The GSV pool is not associated with the TfR and does not cycle to the plasma membrane directly in the basal state. A different model suggests that GLUT4 does not go to the TGN before entering GSV's, but instead travels directly from TfR-containing endosomal membranes directly into GSV's (18). In this model, the GSV pool is relatively static in the basal state. How GLUT4 gets into GSV's is still a matter of debate, with some recent studies have suggested that GLUT4 is transported immediately to GSV's without first going to the plasma membrane, and that this transport requires GGA (Golgi-localized, gamma-ear-containing, Arf-binding proteins)(19), which do not directly bind GLUT4. Another feature of GSV's is that they are cell-type specfic and developmentally regulated (20, 21).

In both models, the GLUT4 in GSV's colocalizes with the insulin-responsive aminopeptidase (IRAP) and somewhat with vesicle-associated membrane protein 2 (VAMP 2) in a perinuclear pool. It is thought that this pool is responsible for insulin-

induced 10-fold increase in cell surface GLUT4 and glucose uptake. Both models predict that in response to insulin, GSV's can translocate directly to the plasma membrane. In response to insulin, the GSV's rapidly redistribute from their perinuclear location to the plasma membrane. There is increased exocytosis and, to a lesser extent, decreased endocytosis of GLUT4-containing vesicles (15), although recent studies have asserted that the decrease in endocytosis is more important than previously believed (16).

Insulin signaling

The early steps in insulin signaling and the ways in which insulin affects GLUT4 trafficking are well known (for review, see (22)). Insulin binds the tyrosine kinase insulin receptor, causing autophosphorylation of the receptor and recruitment of the insulin receptor substrate 1 (IRS-1) and phosphorylation of IRS proteins. These in turn activate phosphatidylinositol 3-kinase (PI-3K), which catalyzes the formation of phosphatidylinositol (3,4,5)-triphosphate, activating phosphoinositide-dependent kinase 1 (PDK1). PDK1 phosphorylates various Akt's, but Akt2 is likely the most relevant for GLUT4 trafficking (23). Akt2 in turn inactivates the Akt substrate AS160, which leads to the activation of various Rab proteins, including Rab10 (24) and Rab14. A second pathway originating at the insulin receptor involves phospholipase C gamma (PLC-gamma) and the atypical protein kinase C beta (PKCß). The role that this pathway plays in GLUT4 trafficking, if any, is unclear (25). However, a PI-3K-dependent pathway which activates the atypical protein kinases zeta and lambda does appear to play a role in GLUT4 trafficking (25, 26). Finally, a third PI-3K independent pathway was more recently identified which involves CAP/APS and the eventual activation of TC10 (27). Of the above, the most relevant pathway for GLUT4 trafficking is believed to be the PI-

3K pathway, which is thought to activate fusion of GSV's with the plasma membrane. For example, the administration of wortmannin (a PI-3K inhibitor) to insulin-treated cells results in accumulation of GSV's at the cell periphery, but not fusion with the plasma membrane (28). It is also believed that PI-3K/AS160 pathway may be involved in the initial movement of GSV's from their perinuclear location to the cell surface, as AS160 is associated with GSV's in the absence, but not presence, of insulin (29). The TC10 pathway, however, may also be involved in stimulating the translocation of GSV's (27). For example, Kif5B, a kinesin motor protein, is involved in mobilizing GLUT4, but mobilization from the perinuclear region was independent of PI-3K, suggesting that it is regulated by a separate pathway (30).

A different theory about the development of insulin resistance is that it arises from defects early in insulin signaling. There is significant evidence that defective mitochondrial function causes elevated intracellular triglycerides, which activate enzymes such as PKC theta (for review, see (31)). These PKC's lead to serine/threonine phosphorylation of IRS proteins, which causes their defective tyrosine phosphorylation and early degradation (32).

The final steps in GLUT4 vesicle trafficking are less well known. Specifically, the immediate proteins that govern the sequestering of GSV's within the cell in the absence of insulin, as well as those that mediate GSV transport to the plasma membrane in the presence of insulin, remain to be defined. The final step in insulin-responsive GLUT4 trafficking to the cell surface (as opposed to the constitutive trafficking to the cell surface) involved fusion of GSV's with the plasma membrane. This step is believed to involved the t-SNAREs syntaxin4 and SNAP23 as well as the v-SNARE VAMP2 (for

review, see (33)). In addition, directing GSV's to their proper docking sites seems to require the exocyst complex (34). Some of these steps might be regulated by insulin. For example, Koumanov *et al.* used an *in vitro* assay in which they reconstituted fusion of GLUT4 vesicles with the plasma membrane and showed that insulin could stimulate fusion 8-fold, suggesting that this is the rate-limiting step in GLUT4 (35). Futhermore, there is still some question about whether the GLUT4 protein itself needs to be further activated to achieve optimal glucose transport, as there are examples of a dissociation between glucose uptake and GLUT4 at the cell surface. However, it is not clear whether these final mechanisms are in fact rate limiting, since disruption of the mechanisms that retain GLUT4 intracellularly can lead to dramatic increases in cell surface GLUT4, suggesting that in the intact cell, fusion might be more limited by the availability of GSV's to fuse (see below).

GLUT4 regulating proteins

Given the importance of GLUT4 trafficking in type 2 diabetes, there have been recent efforts to determine which proteins associate with GLUT4 in an insulin-responsive manner. Several proteins have been shown to bind to GLUT4 directly. These include Daxx, Ubc9, and aldolase, which bind to the C-terminal domain GLUT4. Daxx may be responsible for the SUMOylation of GLUT4. However, these interactions are not affected by insulin. A recent approach to identifying proteins which affect GLUT4 trafficking involved a stable CHO cell line expressing GLUT4 tagged with both green fluorescent protein (GFP) at its C-terminus and myc in an extracellular loop (36). By surface labeling these cells with fluorescent anti-myc antibody, it was then possible to compare the

relative fluorescence and determine the proportion of GLUT4 at the plasma membrane relative to total GLUT4. The cells were then infected with a 3T3-L1 cDNA library expressed in a retroviral vector. This approach identified an 852-nucleotide sequence that encoded the C-terminus of a 60 kDa protein which was named TUG (for 'tether, containing a UBX domain, for GLUT4). This C-terminal fragment was found to significantly reduce the insulin-induced elevation of cell surface GLUT4. This effect was also observed in 3T3-L1 adipocytes expressing the C-terminal fragment of TUG. On the other hand, when full length TUG was expressed in 3T3-L1 adipocytes, the opposite effect was observed: in response to insulin, GLUT4 translocated to the plasma membrane more rapidly and with greater initial magnitude. It was therefore likely that the truncated form of TUG acts as a dominant negative mutant.

In the same study, it was demonstrated that in cotransfected HEK 293 cells, TUG immunoprecipitation pulled down GLUT4, but very little GLUT1, despite the significant homology between GLUT4 and GLUT1. Moreover, in coimmunoprecipitation experiments in 3T3-L1 cells, endogenous TUG binding to GLUT4 was reduced in an insulin-responsive manner (36). This latter finding is important because it is the only protein which has been shown to do this, suggesting that the interaction between TUG and GLUT4 regulates a key step in the translocation of GLUT4.

Typically, discovering regulators of proteins involves either genetic or biochemical approaches. GLUT4 has proven to be a challenge in both regards. Genetics has proven difficult because GLUT4 does not naturally occur in yeast, making it impossible to do mutagenesis studies and screen for abnormal regulation. Biochemical approaches have also been difficult because of the complicated topology of GLUT4.

Because of its twelve transmembrane domains, GLUT4 two-hybrid screens using whole protein have not been possible. Moreover, purification of the whole protein via pull-down is complicated by solubilization conditions and the length of time involved, which can lead to dissociation or degradation of interacting proteins. The discovery of TUG was essentially the result of a genetic screen which circumvented these obstacles.

A more recent study demonstrated that small interfering RNA (siRNA)-mediated knockdown of TUG levels resulted in a dramatic increase in GLUT4 at the plasma membrane in the basal state, a phenotype which was rescued by siRNA-resistant TUG (37). The C-terminal fragment of TUG had a similar phenotype, a finding which supported the idea that it was indeed acting as a dominant negative and the idea that one of the natural functions of TUG is to retain GLUT4 intracellularly. TUG itself is primarily cytosolic, with a small membrane-bound fraction. It was shown directly by microscopy that the absence of TUG specifically mobilizes the perinuclear, insulin-responsive pool of GLUT4. The same study also demonstrated an interaction between TUG and the cytosolic loop of GLUT4 (36).

The fact that the absence of TUG can cause GLUT4 to be targeted to the plasma membrane in the absence of insulin and that overexpression of TUG can cause the an enlargement of the insulin responsive pool of GLUT4 (36) and increase GLUT4 in intracellular, TfR-negative membranes (37) suggests that TUG plays a role in the signaling pathway between insulin and GLUT4 which is not only required but also can not be compensated for by a redundant pathway. It also suggests that TUG is involved in a rate-limiting step, which are often regulated.

Domains in GLUT4 that affect its trafficking

One important point about GLUT4 trafficking is that GLUT4 itself is likely required for tethering of GSV's, and that this effect is not mediated by an entirely independent mechanism. In fact, in GLUT4-null adipocytes, IRAP is not properly sequestered inside the cell but redistributes to the plasma membrane, suggesting that would-be glucose storage vesicles are improperly tethered and constitutively delivering their cargo in the absence of GLUT4 (38). There is some contradictory data to suggest that IRAP itself might be involved in tethering of GSV's via a binding protein p115 (39, 40). Nevertheless, direct mutations in or deletion of various domains within GLUT4 itself can cause its improper trafficking to the plasma membrane. Whether this improper trafficking is of the GLUT4 protein alone or of the entire GSV is still a matter of dispute.

GLUT4 contains twelve transmembrane domains. The N-terminus, the C-terminus, and the large loop between transmembrane domains 6 and 7 are all located on the cytosolic face of the protein. All three of these domains have been found to be involved in its trafficking. Many of the important domains have been discovered based on searching for nonhomologous areas between GLUT4 and GLUT1. For example, the C-terminal domain of GLUT4, and in particular a Leu^{489}-Leu^{490} which is not found in GLUT1, has been found to be important for proper endocytosis of the transporter (41). It has been demonstrated that the C-terminus of GLUT4 shows an increase in antigenicity in response to insulin (28), which has given rise to speculation that this effect is mediated by the dissociation of binding protein(s). As noted above, several proteins have been observed to bind to the C-terminus, but none in an insulin-responsive fashion. It has also been known for over a decade that the Phe-Gln-Gln-Ile (FQQI) motif near the N-terminus

of GLUT4 is important for the proper trafficking and intracellular retention of the protein. Early experiments showed that expressing this motif near the N-terminus of GLUT1 led to intracellular retention of GLUT1 (42). One problem with these earlier studies, however, is that expressing this motif may have had effects on GLUT4 at multiple steps in the recycling pathway. Since both the dileucine and FQQI motifs are also proposed to be involved in GLUT4 endocytosis (16, 41), it was difficult to separate insulin's effects on tethering from its effects on the endocytic arm.

This confounding variable was reasonably well avoided by electroporating adipocytes and looking only at the trafficking of newly synthesized GLUT4. Using transient transfections in the 3T3-L1 adipocyte cell model, Khan *et al*. showed that by swapping various domains of GFP-labelled GLUT4 and GLUT1, they could determine by microscopy the relative importance of these domains to newly synthesized GLUT4 localization in the basal state and in response to insulin (43). Replacement of either the GLUT4 N-terminal domain or the GLUT4 cytosolic loop with the corresponding GLUT1 domains led to targeting of GLUT4 to the plasma membrane, not to GSV's, in the basal state. Furthermore, insulin had very little effect on this localization. On the other hand, substitution of the GLUT4 C-terminal domain with the GLUT1 C-terminal domain had very little effect on GLUT4 trafficking or insulin responsiveness. Conversely, replacement of the GLUT1 N-terminal domain, cytosolic loop, or C-terminal domain with the corresponding domains of GLUT4 did not recapitulate insulin responsiveness in GLUT1. However, double replacement of both the N-terminal domain and cytosolic loop allowed GLUT1 to become insulin-responsive. From this, the authors concluded that the GLUT4 N-terminal domain and cytosolic loop are separately required, and together are

sufficient, for correct basal and insulin-responsive GLUT4 trafficking. The study then scanned specific residues within the N-terminal domain and found that mutating any of four of these residues (Phe^{8}, Ile^{11}[both within the FQQI motif], Asp^{12}, or Gly^{13}) significantly reduced the intracellular retention of GLUT4.

Although the study of these chimeras is based on the assumption that GLUT1 is not insulin responsive, this is somewhat of an oversimplification. Using lentiviral short-hairpin RNA (shRNA) to dramatically reduce GLUT4 levels in 3T3-L1 adipocytes, it has been shown that there is remaining insulin-responsive glucose uptake, which is attributable to GLUT1 (44). Nevertheless, in their assay, which used exogenous GFP-tagged GLUT1, Khan *et al.* did not observe that GLUT1 was sensitive to insulin, which may indicate some level of insensitivity of their assay, different properties of the tagged GLUT1, or possibly even compensatory mechanisms in the lentiviral experiments, such as GLUT1 improperly targeting to insulin-responsive GSV's in the absence of GLUT4.

Since disrupting the GLUT4 N-terminal domain or cytosolic loop gave a phenotype very similar to that of cells expressing a TUG dominant negative mutant and of cells in which TUG expression levels were reduced by siRNA, and since TUG has already been shown to interact with the cytosolic loop of GLUT4, we postulated that TUG might also interact with the GLUT4 N-terminus. If TUG and GLUT4 share a common, nonredundant pathway, then the presence of a defect in either could lead to defective GLUT4 trafficking, and the most direct way for them to share the same pathway would be to interact with each other.

Statement of purpose, specific hypothesis and specific aims

My primary hypothesis is that TUG interacts with the N-terminal domain of GLUT4, a domain critical for proper GLUT4 trafficking. A secondary hypothesis is that this interaction is what is disrupted by point mutations in the GLUT4 N-terminal domain which have previously been shown to cause defective GLUT4 trafficking.

Aim 1. To test whether TUG interacts with the GLUT4 N-terminal domain. It has already been demonstrated that TUG interacts with the GLUT4 cytosolic loop (37). We hypothesize that since the absence of an N-terminal domain in GLUT4 causes a phenotype of GLUT4 localization identical to that seen in cells expressing reduced TUG or expressing a TUG dominant-negative mutant (36, 37, 43), TUG might also interact with the GLUT4 N-terminal domain. This hypothesis is tested using fusion proteins of GST with three domains of GLUT4 to pull down TUG from the lysates of TUG-overexpressing cells.

Aim 2. To determine whether TUG interacts with the wild-type GLUT4 N-terminal domain but not with a mutant form known to cause defective trafficking. We further hypothesize that the mutations in the N-terminal domain of GLUT4 which cause defective intracellular retention of GLUT4 (43) also disrupt TUG's interaction with the GLUT4 N-terminus. This hypothesis is tested using synthetic wild-type and mutant GLUT4 N-terminal peptide to pull down TUG from the lysates of TUG-transfected cells.

Materials and Methods

Note: All of the experiments were carried out by the primary author alone.

Reagents

Full-length FLAG-tagged TUG and TUG L1-C (N-terminally truncated TUG containing amino acids 165-550 only) in pcDNA 3.1 vector were provided by Chenfei Yu in the Bogan lab. Plasmids encoding the GLUT4-GST fusion proteins were a generous gift from Jian Li and Victor Hsu (Harvard University). DNA sequences were verified as described below. Synthetic biotin-labeled wild-type and mutant GLUT4 N-terminal peptides (MPSGFQQIGSEDGEPPQQRVTGTLVGG-biotin and MPSG**A**QQ**A**GSE**AA**EPPQQRVTGTLVGG-biotin, respectively) were synthesized by the W.M. Keck Facility at Yale. Note that the mutant peptide contains residue changes to alanine at F5, I8, D12, and E13.

Expression and purifiation of GLUT4-GST fusion proteins

The BL21 *E. coli* bacterial strain was transformed with pGEX-4T3 vector containing GLUT4 N-terminus (amino acids 1-24), middle loop (amino acids 223-287), or C-terminus (amino acids 467-509), each fused to GST, or with vector containing GST alone, and bacteria were incubated overnight on ampicillin-containing agar plates. Colonies were selected and incubated in ampicillin-containing Luria Broth until the

cultures reached an OD_{600} of 0.4, at which point protein expression with induced with 100μM isopropyl ß-D-1-thiogalactopyranoside (IPTG) for 6 hours. An aliquot of each culture was removed prior to stimulation for DNA extraction (QIAprep miniprep kit, Qiagen) and confirmatory sequencing by the W.M. Keck Facility. Bacterial cultures were centrifuged and pellets were lysed in ice-cold NETN buffer (100mM NaCl, 1mM EDTA, 20mM Tris-Hcl, pH 8.0, and 0.5% Nonidet P-40) containing lysozyme, followed by sonication in 10 sec bursts for 2 min (at 4°C). Lysate was centrifuged at 12,000 x g for 1 h, and the supernatant was removed and incubated overnight with glutathione-Sepharose beads (Amersham). The beads were washed 3x with ice-cold NETN, then resuspended in a small volume of NETN. To assess the quality of expression and purification, aliquots of the beads were eluted in SDS-containing sample buffer, run on SDS-PAGE gels, and Coomassie stained.

Expression and purification of TUG and its truncation mutant in HEK 293 cells

HEK 293 cells were cultured and transfected with FLAG-tagged full-length TUG, TUG L1-C (a.a. 165-500), or empty pcDNA 3.1 vector using a cationic lipid transfection reagent (Lipofectamine 2000; Invitrogen). Cells were lysed in ice-cold NETN buffer supplemented with 1% Nonidet P-40 (NP-40) and a protease inhibitor (PI) cocktail (Complete Protease Inhibitor Cocktail; Roche) for 30 min and centrifuged at 14,000 *x g* to remove unsolubilized material. Aliquots of lysate were analyzed by SDS-PAGE and western blotted with an anti-TUG antibody (as described below) to verify expression levels and adjust the volume of lysate used for pull-down experiments.

Pull-down with GLUT4-GST fusion proteins

Lysates from 293 cells transfected with full-length TUG, TUG L1-C, or vector alone were diluted 35-fold in PBS (20mM sodium phosphate, 150mM NaCl) + 1% NP-40 + PI cocktail and precleared with GST bound to glutathione-agarose beads for at least 3h. The diluted lysates were centrifuged and supernatants were incubated overnight at 4°C with the GLUT4-GST fusion proteins bound to glutathione-agarose beads, controlling for the volume of beads. After incubation, the samples were centrifuged and the beads were washed 3x in PBS + 1% NP-40 + PI cocktail. Samples were eluted by boiling in SDS sample buffer, separated by SDS-PAGE, and western blotted with anti-TUG antibody as described below.

Pull-down with synthetic GLUT4 N-terminal peptides

Synthetic biotin-labeled wild-type and mutant GLUT4 N-terminal peptides were incubated with streptavidin-agarose beads (Invitrogen) for at least 3h. Concurrently, lysates from 293 cells transfected with full-length TUG or vector alone were diluted 35-fold in PBS + 1% NP-40 + PI cocktail and precleared for at least 3h with streptavidin-agarose beads alone. Samples were centrifuged, and the supernatant of the latter samples (i.e., precleared lysates) were incubated overnight at 4°C with the peptide-bound beads. The beads were then washed 3x in PBS + 1% NP-40 + PI cocktail. Samples were eluted in SDS sample buffer, separated by SDS-PAGE, and western blotted as described below.

SDS-PAGE and Western blotting

Samples were eluted from beads by boiling in sample buffer containing 1% SDS and 2.5% ß-mercaptoethanol, then run on 4-12% NuPAGE gels in MOPS buffer (Invitrogen) and transferred to nitrocellulose membranes. The membranes were blocked for at least one hour in 5% milk, then incubated in a polyclonal anti-TUG primary antibody (affinity purified rabbit antiserum raised to C-terminus of TUG) at a dilution of 1:1000 in 5% milk. Following 3 washes with PBST, membranes were incubated in HRP-conjugated goat anti-rabbit secondary antibody (Jackson Laboratory) at a dilution of 1:100,000 in 5% milk, then washed again in PBST (PBS + 0.2% Tween). Bands were visualized using a chemiluminescent HRP subtrate (SuperSignal West Pico; Pierce).

Results

GST fusion proteins containing the N-terminal domain, large cytosolic loop, or C-terminal domain of GLUT4 were expressed in bacteria and purified onto glutathione-agarose beads as described in Methods. To determine whether the fusion proteins were properly expressed and purified, aliquots were run on SDS-PAGE and gels were Coomassie-stained (Figure 2). All the proteins, including GST alone, migrated at their expected molecular weights (~26 kDa for GST alone, ~28 kDa for GST-GLUT4 N-terminus, ~32 kDa for GST-GLUT4 loop, and ~30 kDa for GST-GLUT4 C-terminus). The GST-GLUT4 cytosolic loop did contain some lower molecular weight bands, likely representing degradation products or possibly incomplete transcription products. In the

other samples, no other significant bands were visualized, indicating that the purification process was relatively efficient.

To determine whether TUG could interact with these domains of GLUT4, the fusion proteins were incubated with lysates from HEK 293 cells transfected with either full length TUG, with a truncated form of TUG lacking amino acids 1-164 from the N-terminus, or with vector only. The eluates from the beads were western blotted with an antiserum raised to the C-terminus of TUG (Figure 3). The fusion proteins were balanced based on their respective concentrations, and equal loading was verified by Ponceau-S staining of the membrane (data not shown). In the lysate-only lane, full-length TUG runs at its expected molecular weight of ~60kDa, with a secondary lower molecular weight band seen, possibly representing a incompletely translated product or a degradation product. Although very little full-length TUG is pulled down by GST alone (lane 5), the GLUT4 N-terminal domain and cytosolic loop pulled down significant amounts of full-length TUG. The GLUT4 C-terminal domain also pulled down more than GST alone, but significantly less than the other two domains tested. These results were observed twice, with two separate preparations of GLUT4-GST fusion proteins.

Interestingly, the results from pull-downs of N-terminally truncated TUG with the three GST fusion proteins were very different (Figure 3, lanes 6-10). Although background binding to GST is very similar to the binding seen with full-length TUG, neither the GLUT4 N-terminal domain, cytosolic loop, or C-terminal domain pull down any more truncated TUG than GST alone. This suggests that the site in TUG required for binding is within its N-terminal 164 amino acids.

Although HEK 293 cells do contain endogenous TUG, no TUG is seen in the straight lysate from vector-transfected cells (Figure 3, lane 11), let alone in the pull-down lanes (lanes 12-15). This supports the conclusion that the bands seen in lanes 2-5 represent full-length transfected TUG and not a cross-reactive endogenous protein. Some endogenous TUG can be seen in the vector lysate lane at longer exposures (data not shown); however, the band is very faint, likely due to the small amounts of lysate needed, and endogenous TUG would be expected to degrade relatively quickly in the pull-down lanes. Thus, no conclusions can be currently be drawn about the binding of endogenous TUG to the GLUT4-GST fusion proteins.

Since these results indicated that TUG could indeed be pulled down by the N-terminal domain of GLUT4, we conducted a second set of pull-down experiments with synthetic biotinylated GLUT4 N-terminal peptide and a mutant peptide containing alanine substitutions at 4 of its 27 amino acids. Lysates from 293 cells transfected with full-length TUG or vector alone were precleared with streptavidin-agarose beads. The biotinylated peptides were also incubated with streptavidin-agarose beads, and then the peptide-bound beads were incubated with the precleared lysates. The samples were eluted in SDS-containing sample buffer and the eluates were separated by SDS-PAGE and western blotted with the anti-TUG antiserum (Figure 4).

As can be seen, wild-type GLUT4 N-terminal peptide pulled down a protein that runs at a slightly higher molecular weight than full-length TUG (Figure 4, lane 2). This protein can be faintly seen in the straight lysate (lane 1). Moreover, this interaction is very strong, since the band pulled down is much stronger than that seen in the lysate. Since the lysate lane contains approximately 5% of the lysate used in the pull-down

lanes, and since the pull-down lane (lane 2) has at least 10 times the amount seen in the lysate lane, we estimate that at least half of that ~64kDa protein is pulled down the by wild-type GLUT4 N-terminal peptide. Furthermore, the band is very specific to the wild-type GLUT4 N-terminal peptide pull-down, as it is not pulled down by the mutant peptide (lane 3) or streptavidin beads alone (lane 4). The ~64 kDa protein also does not represent a contaminant in the synthetic wild-type preparation either, since it is not observed in the wild-type pull-down from vector-transfected lysates (lane 6). This result was observed on three separate occasions with two separate sets of transfections.

Interestingly, the results for the ~60kDa band, where full-length TUG normally migrates, were quite different. In the streptavidin only pull-down (lane 4), we see that there is quite a bit of background binding. Despite the use of various detergents including NP-40 and Triton X-100, this background binding was still present. This band is almost certainly the transfected full-length TUG as it is not seen in the pull-down from vector-transfected 293 cells. We actually saw lower levels of binding of the full-length TUG in both the wild-type and mutant peptide pull-downs (lanes 2 and 3). Furthermore, the wild-type pull-down had even less ~60kDa full-length TUG than the mutant peptide pull-down. These results will be examined in more detail below.

Discussion

Our first set of experiments show that TUG can interact with the GLUT4 N-terminal domain and its large cytosolic loop. In fact, the GLUT4 N-terminal domain pulled down even more full-length TUG than the cytosolic loop did. This binding is specific. It is also not seen between TUG L1-C and any of the GLUT4 domains. This

suggests that the binding sites for both the GLUT4 N-terminus and cytoplasmic loop are within the first 164 amino acids at the N-terminus of TUG. All three GLUT4 domains tested lie on the cytoplasmic face of the vesicle. Since TUG is primarily a cytoplasmic protein, this binding is topologically consistent.

It has previously been shown that, *in vitro*, TUG binds to the large cytosolic loop of GLUT4 but not GLUT1(37). Our results are consistent with this finding. It has also been previously shown that truncating TUG at its N-terminus leads to poor insulin response and loss of *in vitro* binding to GLUT4 (36). Our results are also consistent with these finding as well. Our novel finding from this first set of experiments is that TUG binds specifically to the N-terminal domain of GLUT4, which is known to be critical for proper GLUT4 trafficking.

Our second set of experiments were more difficult to interpret due to the presence of the higher molecular weight (~64kDa) band pulled down by GLUT4 N-terminal wild-type, but not mutant, peptide. This higher molecular weight band could represent full length TUG still bound to the biotinylated peptide through a noncovalent, SDS-resistant interaction. Alternatively, it could represent a modified form of full length TUG, such as a phosphorylated TUG, which would be intriguing because it would suggest that TUG binding is somehow controlled by phosphorylation. The band could also represent a modified form of a lower molecular-weight product of TUG, such as a C-terminal fragment covalently modified by ubiquitin or the ubiquitin-like modifier SUMO. Finally, the ~64kDa band could be a protein that cross-reacts with the TUG antibody. However, since the band is not seen in the vector-transfected negative controls, in order for this to be a different protein from TUG, either its expression would have to be induced by the

transfected TUG or it would somehow require the presence of TUG to allow it to interact with the GLUT4 N-terminus and be pulled down. The likelihood that a protein exists that not only satisfies one of these two possibilities, but also has such a close molecular weight to TUG and is detected by an anti-TUG antibody, is pretty low. Nevertheless, it is a possibility that cannot be ruled out until the protein is identified.

We currently suspect that the ~64 kDa band is in fact full-length TUG bound by the biotinylated peptide. There are several reasons for this. First, the shift is within the margin of error for what one might expect by adding the 27 amino acids (~3 kDa) to full-length TUG (~60 kDa). It is entirely possible for a coiled-coil interaction, for example, to be strong enough to withstand boiling in SDS. Second, if some post-translational modification were required for TUG to bind the GLUT4 N-terminal domain, then we would have seen the ~64 kDa band in the GST-GLUT4 pull-downs experiments as well (Figure 3). Finally, this could explain why the 60 kDa band is not enhanced in the wild-type peptide pull-down (Figure 4, lane 2) relative to beads alone (lane 4). If one were to "add" the ~64 and 60 kDa bands, it would be consistent with the results of the GST-GLUT4 fusion protein experiments, in which TUG binds the GLUT4 N-terminal domain much more strongly than it binds the negative control. The only inconsistency with this interpretation is that the ~64 kDa band is also seen, albeit faintly, in the lysate-only lane.

Looking forward, there are a number of directions I would like to pursue. One of the main drawbacks of using pull-downs from total lysates is that even though it suggests that there is a direct interaction between GLUT4 and TUG, it does not prove that this interaction is direct, since another protein (or proteins) might be linking the two together. One direction would thus be to test the interaction between GLUT4 and TUG directly

using recombinant proteins only and not lysates. I did attempt to do this using *in vitro* transcription/translation of radiolabeled full length TUG, followed by pull-downs with the GLUT4-GST fusion proteins. However, I was unable to see any binding above background binding to GST alone (data not shown). I also expressed a full-length TUG-GST fusion protein, cleaved the TUG from the GST with thrombin, and attempted to pull the TUG protein down with the GST fusion proteins, but I was again unable to see any specific binding (data not shown). These experiments could indicate that TUG requires the full translational machinery of the cell for proper folding, that TUG requires some post-translational modification before it can bind, or that some additional protein(s) present in lysates are required for the interaction to occur (directly or indirectly). However, since it has previously been reported that a GST-GLUT4 cytosolic loop fusion protein can pull down full length TUG in such an *in vitro* environment (37), the negative result might simply indicate that the binding reaction conditions that I used were not ideal. This direction is thus worth pursuing again, since being able to test the protein-protein interactions outside of the cell would eliminate some confounding variables and make it easier to test various mutantions in TUG and their effects on binding.

A second line of inquiry would be to purify and identify the ~64 kDa band pulled down by the wild-type GLUT4 N-terminal peptide. In general, there are two approaches to the identification of this band: a candidate-based approach or a more direct approach. As far as the candidate-based approach, the first and most obvious candidate is TUG itself, whether it be full-length TUG or a modified product. I would like to repeat the experiment and western blot with antibodies to the N-terminus or to an internal domain of TUG to determine whether this band represents full length TUG, or possibly a modified

(or dimerized) C-terminal fragment. The latter might be detected by the antibody to an internal epitope but not by the antibody to the N-terminus. I would also repeat the experiment and blot with streptavidin-HRP. A positive result would suggest that the ~64 kDa band is TUG strongly bound to the biotinylated wild-type GLUT4 peptide. The ~64 kDa band could also be tested for the presence of modifications such as phosphorylation (using anti-phosphoserine, phosphothreonine, or phosphotyrosine antibodies), ubiquitination, or SUMOylation (using anti-ubiquitin or anti-SUMO antibodies, respectively). In a similar fashion, we could repeat the experiment and probe for known GLUT4-binding proteins of a similar molecular weight, such as GTB70 (glucose transporter-binding protein of 70 kDa)(45). However, very few proteins fit this description, since most identified GLUT4-binding proteins are either significantly smaller (e.g., Ubc9 [18kDa], L-3-hydroxyacyl-coA-dehydrogenase [34kDa]) or larger (e.g., Daxx [110kDa] or aldolase [158kDa]).

The more direct and less biased approach would involve mass spectometry. Given that a large percentage of the ~64 kDa protein was pulled out of lysates by wild-type GLUT4 N-terminal peptide, and the fact that relatively little starting material was needed to visualize the band on Western blot, by scaling up the reagents used, we could likely achieve quantities of protein sufficient for visualization by silver stain or even Coomassie staining. Furthermore, Coomassie or silver-staining the gels after scaling up would allow us to determine whether any other proteins are being pulled down specifically by the N-terminal wild-type peptide. If no other proteins are seen, it is likely that the interaction between GLUT4 and the ~64kDa band is direct and does not require linking proteins.

Even if the ~64kDa band does not represent a modified form of TUG, it is still of interest because it interacts so strongly with the wild-type GLUT4 N terminus but not at all with the mutant form, which contains mutations that are known to disrupt GLUT4 trafficking. The same applies to any other proteins that are pulled down and visualized by Coomassie or silver staining, since these could potentially represent other members of an insulin-responsive GLUT4-bound complex.

In order to determine whether the interaction between TUG and GLUT4 occurs under more physiological conditions, full-length GLUT4 with and without N-terminal mutations could be expressed in cells along with full-length TUG, followed by coimmunoprecipitation experiments. This could be done in HEK293 cells, or alternatively, directly in 3T3-L1 adipocytes. Although the latter approach would be more physiologically appropriate, it is complicated by the fact that 3T3-L1 adipocytes contain endogenous GLUT4. This limitation could be overcome by reducing endogenous GLUT4 using lentiviral shRNA knockdown, then transfecting with either a knockdown-resistant wild-type or mutant GLUT4, followed by TUG or GLUT4 coimmunoprecipitation. We would thus be able to see if the cytosolic loop and C-terminal domains of full-length GLUT4 are able to compensate for the GLUT4 N-terminal mutations, or whether GLUT4 and TUG no longer interact in the presence of these mutations. This would also indicate the relative importance of the three binding domains of GLUT4.

A separate line of inquiry would be to try to determine the precise site of interaction on TUG. This could be achieved by mutating various subdomains near the N-terminus of TUG, then repeating *in vitro* experiments following the same protocol used to generate Figure 3. Alternatively, it could be achieved in 3T3-L1 adipocytes directly by

following a protocol similar to the one just outlined above. This approach would have the distinct advantage that mutations in TUG could be assessed not only for their effects on GLUT4 binding, but also for their functional consequence on GLUT4 trafficking and glucose uptake.

Once the precise binding sites on TUG are determined, another potential set of experiments would be to use fluorescence resonance energy transfer (FRET) to study the specific insulin signals or other molecules which cause dissociation of TUG and GLUT4. Both proteins could be fluorescently labelled near their respective binding sites, and using inhibitors of the various insulin signaling pathways, TUG-GLUT4 dissociation could be monitored in real-time. This approach would help elucidate which pathways that specifically regulate TUG-GLUT4 dissociation versus other steps in GLUT4 trafficking to the plasma membrane (such as membrane fusion). It would also allow the study of exogenous compounds that directly disrupt this interaction, which would be helpful for drug development.

The goals of the above experiments would be to determine whether TUG and GLUT4 directly interact, whether their interaction(s) occurs naturally in adipocytes, and whether their interaction(s) are physiologically relevant to insulin signaling. A larger goal that could then be pursued is to determine other members (if any) of the GLUT4 complex, how they respond to insulin, and whether they interact abnomally in insulin resistant tissues. This would not only answer the basic scientific questions of the processes governing insulin signaling and GLUT4 trafficking, but it would also provide a target that could ultimately be manipulated pharmacologically. Disruption of the GLUT4 complex through carefully designed inhibitors could lead to targeting of GLUT4 to the

plasma membrane, subsequently reducing circulating plasma glucose levels and helping avoid some of the complications of diabetes.

Elevating GLUT4 levels at the plasma membrane in order to reduce plasma glucose levels and increase insulin sensitivity is a strategy that is supported not only by current models of glucose homeostasis, but also directly by mouse models of GLUT4 overexpression. Overexpression of GLUT4 in skeletal muscle (46) or adipose tissue (47) in mice leads to increased insulin sensitivity and glucose tolerance. Moreover, muscle-specific overexpression of GLUT4 in heterozygous $GLUT4^{+/-}$ mice prevents the insulin resistance and diabetic phenotype that these mice would otherwise develop (48).

In summary, using fusion proteins of GST with either the GLUT4 N-terminal domain, large cytoplasmic loop, or C-terminal domain, we were able to pull full-length TUG down from lysates of cells overexpressing TUG. All three fusion proteins pulled down TUG to some extent, but the N-terminal domain and loop did so most efficiently. Moreover, a truncation mutant lacking the N-terminal 164 amino acids of TUG was not preferentially pulled down by any of these GLUT4-GST fusion proteins over GST alone, suggesting that the binding site on TUG is most likely in these N-terminal 164 amino acids. Using a similar paradigm, we then attempted to pull TUG down from the same lysates using a biotinylated synthetic GLUT4 N-terminal peptide. We found that a ~64 kDa band, which we believe to be a form of TUG, was pulled down by the wild-type N-terminal peptide, but not by a peptide mutated at the four residues shown by Khan *et al.* to be necessary for proper GLUT4 intracellular retention and insulin-responsive trafficking. We conclude that TUG interacts with both the large cytosolic loop and N-terminal domain of GLUT4 and that this latter interaction is disrupted by known

mutations that influence GLUT4 trafficking, providing evidence that this interaction specifically may be critical for both intracellular retention of GLUT4 and insulin-responsive GLUT4 trafficking.

The fact that TUG binding is disrupted by the mutations at the F5, I8, D12, and G13 residues at the N-terminus of GLUT4 provides strong additional evidence for the hypothesis that TUG regulates an insulin responsive step in GLUT4 trafficking. These data also implicate the GLUT4 N-terminus as a second interacting region, in addition to the cytosolic loop, required to bind TUG. Since TUG interacts with at least two noncontiguous domains of GLUT4, this (combined with the fact that TUG is relatively unstable in lysates and endogenous TUG is often degraded in lysates) may possibly explain why TUG was not discovered earlier using biochemical methods.

The exact step in GLUT4 trafficking regulated by TUG is still not known. TUG might be required for the initial sorting of GLUT4 to GSV's, for retaining the GSV's intracellularly in the absence of insulin, or a combination of both of these processes. Alternatively, or perhaps in addition, TUG could be involved in loading GLUT4 onto kinesin motors that transport GLUT4 to the cell surface. In Khan *et al.*, the authors speculate that their results implicate the N-terminus of GLUT4 in the intial sorting event (e.g., from TGN to GSV's). However, it is possible that this effect is on retention of the GSV's. It is not yet known whether the mutated GLUT4 travels to the plasma membrane in GSV's or in a different vesicle. Further studies will be necessary to answer these questions.

The ways in which insulin might modulate TUG are also unknown. The insulin signaling pathways might induce a conformational change in TUG. Alternatively, indulin

might cause phosphorylation, cleavage, or another post-translational modification of TUG. This insulin-induced modification could lead to dissociation of TUG from GLUT4, thus targeting GLUT4 to the plasma membrane, or it could lead to dissociation of all or part of TUG from some unidentified anchor, thus targeting GLUT4 and TUG together to the plasma membrane.

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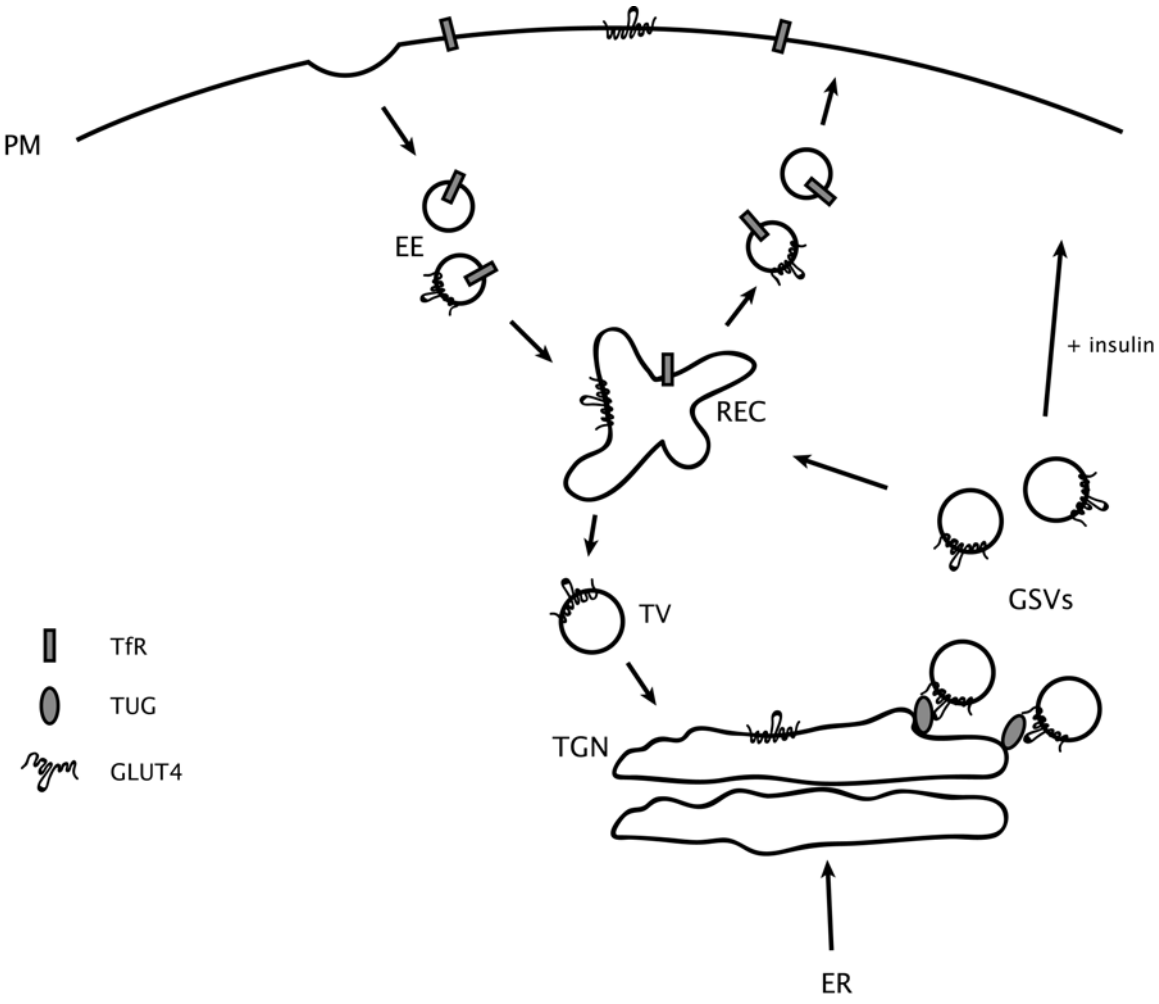
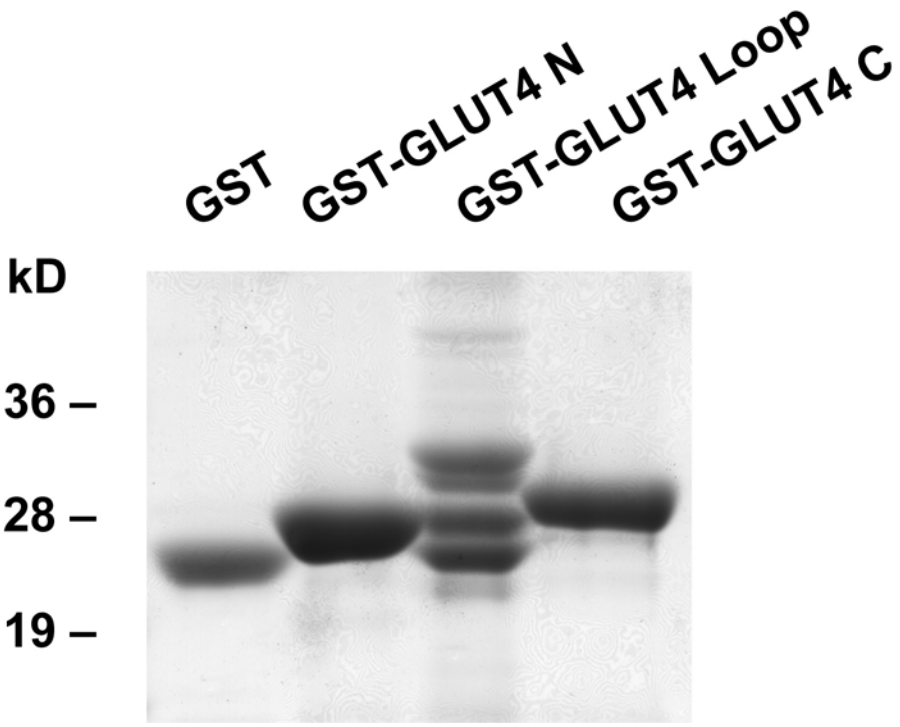
Figure 1: Current model of GLUT4 trafficking



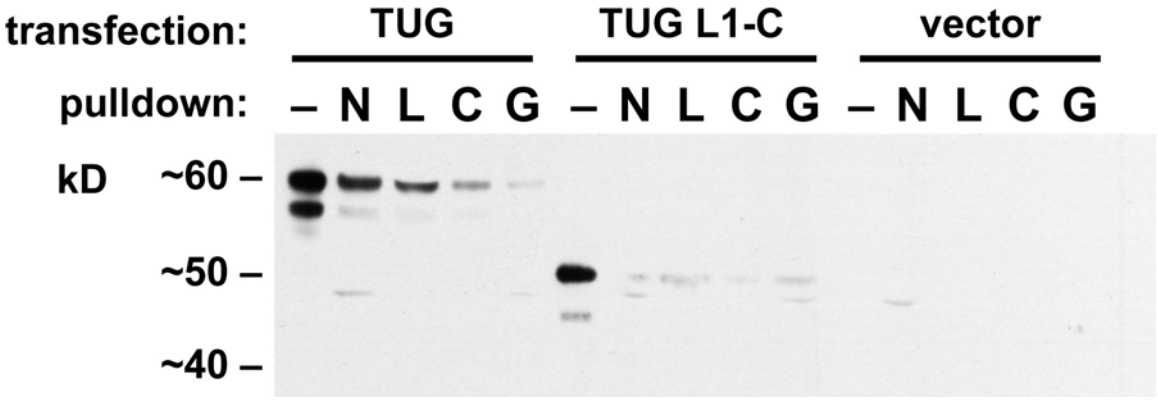
(“PM”: Plasma membrane. “EE”: Early endosomes. “REC”: Recycling endosome compartment. “TV”: Transport vesicles. “GSVs”: GLUT4 storage vesicles. “TGN”: Trans-Golgi network. “TfR”: Transferrin receptor. “GLUT4”: Glucose transporter 4. “TUG”: Tether, containing a UBX domain, for GLUT4. Note that in the absence of insulin, the majority of GLUT4 is intracellular and only partly colocalizes with TfR. Also note that the location of TUG is putative. Adapted in part from Bryant *et al.* (17))

Figure 2: Purification of GST-GLUT4 fusion proteins (Coomassie Stain)



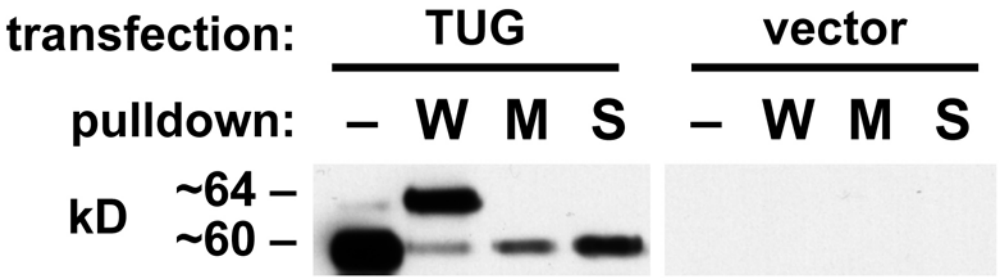
(Legend: “GST”: Glutathione S-transferase. “GST-GLUT4 N”: Fusion protein of GST and GLUT4 N-terminal domain (amino acids 1-24). “GST-GLUT4 Loop”: Fusion protein of GST and GLUT4 large cytosolic loop (amino acids 223-287). “GST-GLUT4 C”: Fusion protein of GST and GLUT4 C-terminal domain (amino acids 467-509))

Figure 3: Full length TUG, but not truncated TUG, is pulled down by the N-terminal, cytosolic loop, and C-terminal domains of GLUT4



(Legend: “TUG”: HEK 293 cells transfected with full-length TUG. “TUG L1-C”: HEK 293 cells transfected with TUG lacking its 164 N-terminal amino acids. “vector”: HEK 293 cells mock-transfected with empty vector alone. “–“: no pull-down (lysate only, approximately 5% of pull-down input). “N”: GLUT4 N-terminal domain. “L”: GLUT4 large cytosolic loop. “C”: GLUT4 C-terminal domain. “G”: GST alone)

Figure 4: Wild-type, but not mutant, GLUT4 N-terminal peptide pulls down a TUG-positive band



(Legend: “TUG”: HEK 293 cells transfected with full-length TUG. “vector”: HEK 293 cells mock-transfected with empty vector alone. “–“: no pull-down (lysate only, approximately 5% of pull-down input). “W”: wild-type GLUT4 N-terminal peptide. “M”: mutant GLUT4 N-terminal peptide. “S”: Streptavidin-agarose beads alone.)