Entry of Newly Synthesized GLUT4 into the Insulin-responsive Storage Compartment Is Dependent upon Both the Amino Terminus and the Large Cytoplasmic Loop*

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We have recently reported that following initial biosynthesis, the GLUT4 protein exits the Golgi apparatus and directly enters the insulin-responsive compartment(s) without transiting the plasma membrane (Watson, R. T., Khan, A. H., Furukawa, M., Hou, J. C., Li, L., Kanzaki, M., Okada, S., Kandror, K. V., and Pessin, J. E. (2004) EMBO J. 23, 2059–2070). To investigate the structural motifs involved in these initial sorting events, we have generated a variety of loss-of-function and gain-of-function GLUT4/GLUT1 chimera proteins. Substitution of the GLUT4 carboxy-terminal domain with GLUT1 had no significant effect on the acquisition of insulin responsiveness. In contrast, substitution of either the GLUT4 amino-terminal domain or the large cytoplasmic loop between transmembrane domains 6 and 7 resulted in the rapid default of GLUT4 to the plasma membrane with blunted insulin response. Consistent with these findings, substitution of the amino-terminal, cytoplasmic loop, or carboxyl-terminal domains individually into GLUT1 backbone did recapitulate normal GLUT4 trafficking. Similarly, dual substitutions of the GLUT1 amino and carboxyl termini with GLUT4 domains or the combination of the cytoplasmic loop plus the carboxyl terminus failed to display normal GLUT4 trafficking. However, the dual replacement of the amino terminus plus the cytoplasmic loop of GLUT4 in the GLUT1 backbone resulted in a complete restoration of normal GLUT4 trafficking. Alanine-scanning mutagenesis of the GLUT4 amino terminus demonstrated that Phe⁵ and Ile⁸ within the FQQI motif and, to a lesser extent, Asp¹⁸⁵/Gly¹³ were necessary for the appropriate initial trafficking following biosynthesis. In addition, amino acids 229–271 in the large intracellular loop between transmembrane domains 6 and 7 functionally cooperated with the amino-terminal domain. These data demonstrate that initial trafficking of GLUT4 from the Golgi to the insulin-responsive GLUT4 compartment requires the functional interaction of two distinct domains.

The facilitative glucose transporters constitute an extensive family of 12 membrane-spanning glycoproteins that are characterized by their different tissue distribution patterns and biochemical properties (reviewed in Refs. 1 and 2). GLUT4 is unique among the facilitative glucose transporters as it is predominantly expressed in adipose tissue and striated muscle (skeletal and cardiac), the sites responsible for postprandial glucose uptake (3–5). The first glucose transporter identified, GLUT1, is responsible for basal glucose uptake with its predominantly plasma membrane localization and wide pattern of tissue expression (5–7). Despite the overall 65% amino acid identity between GLUT4 and GLUT1 with the same predicted secondary structure, general kinetic characteristics, and stereospecificity transport, these two proteins display markedly different intracellular trafficking properties.

In the basal state, GLUT1 is plasma membrane-localized with a relatively small amount found in intracellular membrane compartments (8, 9). In contrast, the majority of GLUT4 is found in various intracellular compartments with only a small amount found at the plasma membrane. Both of these glucose transporter proteins continuously recycle, but the exocytotic rate of GLUT1 is relatively high, whereas the endocytic rate of GLUT4 is relatively low when compared with the rate of endocytosis (10–12). Although insulin stimulation has only a marginal effect on the rate of GLUT1 trafficking, it markedly enhances the rate of GLUT4 exocytosis. Thus, the insulin stimulation of GLUT4 exocytosis accounts for the large increase of GLUT4 at the plasma membrane in the postprandial state. Recent studies have suggested that GLUT1, as well as other constitutively recycling membrane proteins, traffic between the plasma membrane and early endosome compartments. In contrast, GLUT4 is thought to undergo sequestration from these recycling early endosomes by recycling continuously to and from other endosome compartments and/or the trans-Golgi network (13–15). Insulin stimulation results in the exit of GLUT4 from these storage compartments and entry into the constitutively recycling early endosome compartments that contain GLUT1 and other constitutively trafficking proteins such as the transferrin receptor (16, 17).

To identify specific GLUT4 domains that are responsible for its unique trafficking properties, several laboratories have examined the localization of GLUT4/GLUT1 or GLUT4/transferrin receptor chimeras (reviewed in Refs. 5 and 18). However, these studies have resulted in remarkably divergent and inconsistent conclusions. Initial studies suggested that the amino-terminal hydrophilic domain of GLUT4 is both necessary and sufficient for its intracellular sequestration and identified Phe⁵ as a key residue (19, 20). In contrast, other studies reported that the major determinant of GLUT4 localization re-
sides in the carboxyl-terminal sequences, with a dileucine motif at positions 489 and 490 identified as a critical component (21–24). Furthermore, some of these studies showed that point mutations of either Phe or Leu resulted in inappropriate targeting of GLUT4 to the cell surface (19, 23, 25). Alternatively, another study suggested that several non-contiguous domains function cooperatively to correctly target GLUT4 (26). Subsequently, both the amino-terminal and the carboxyl-terminal GLUT4 domains were found not to be responsible for selective intracellular retention but serve as important plasma membrane endocytic domains (27, 28).

More recently, it has been suggested that the amino- and carboxyl-terminal domains are both required for efficient GLUT4 trafficking regulation by functioning at distinct intracellular sorting steps. The amino-terminal "FQ" motif has also been implicated in the sorting of GLUT4 out of early endosomes (29, 30), whereas the Leu motif may contribute to the movement of GLUT4 from the trans-Golgi network to recycling endosomes or from the trans-Golgi network to the GLUT4 storage compartments (GSCs) (30, 31). Moreover, other domains have recently been implicated in the regulated trafficking of GLUT4. In particular, an acidic cluster adjacent to the dileucine motif in the carboxyl terminus (498TELEY-LGP505) has been implicated in intracellular targeting and sorting from recycling endosomes to the insulin-responsive storage compartments and Tyr26 with the terminal residues 505PDEND509 in modulating GLUT4 retention within the GLUT4 storage compartments (31, 32).

Although a few studies have examined the trafficking of GLUT4 following cell surface labeling, the majority have not accounted for the dynamic and continuous trafficking properties of GLUT4. Thus, the analysis of GLUT4 distribution was determined after long term expression in which GLUT4 has equilibrated throughout its entire trafficking pathway. Moreover, many of these studies have utilized deletions or mutations and have inferred the importance of specific motifs from the apparent loss-of-function. To address these issues, we have recently reported that following initial biosynthesis, GLUT4 directly traffic from the secretory membrane system (Golgi/trans-Golgi network) to the specialized GLUT4 storage compartments without transiting the plasma membrane (33). Through the examination of the trafficking of newly synthesized GLUT4, we are able to eliminate the notion of plasma membrane endocytosis and endosome recycling, thus allowing us to selectively identify sequence motifs responsible for the initial entry into the insulin-responsive GLUT4 storage compartments. In this study, we have used both loss-of-function and gain-of-function mutations to conclusively demonstrate that the amino-terminal domain and large cytoplasmic loop are both necessary and sufficient to confer the appropriate trafficking of GLUT4 from the secretory membrane transport system to the GSC.

EXPERIMENTAL PROCEDURES

MATERIALS—Mini-prep DNA and DNA gel extraction kits were purchased from Qiagen Inc. (Valencia, CA). PhTURbo DNA polymerase was obtained from Stratagene (La Jolla, CA). Insulin, dexamethasone, 3-isobutyl-1-methylxantine, bovine serum albumin, and donkey serum were purchased from Sigma. Vectashield was obtained from Vector Laboratories (Burlingame, CA).

PLASMIDS—A pcDNAs-EGFP (enhanced green fluorescent protein) plasmid was prepared by cloning the EGFP cDNA (Clontech) into the NotI-XbaI sites of the mammalian expression vector pcDNA3 obtained from Invitrogen. The wild type GLUT4-EGFP and the wild type GLUT1-EGFP cDNA constructs were prepared by subcloning the full-

FIG. 1. Schematic representation of the GLUT4/GLUT1 chimeras. GLUT4 is represented in dark gray, and GLUT1 is represented in pale gray. The small squares represent the regions exchanged. The corresponding amino acids for GLUT4 and GLUT1, respectively, are: 1–30 and 1–18 for the amino terminus (due to a very low level of expression of original chimera 4–144, the amino acids replaced in this particular case are 1–19 for GLUT4 and 1–7 for GLUT1); 229–284 and 213–268 for the intracellular loop, and 478–509 and 462–492 for the carboxyl terminus. The nomenclature of the chimeras is described under Experimental Procedures. A, GLUT4 and GLUT1 wild type constructs; B, GLUT4/GLUT1 chimeras with GLUT4 backbone (loss-of-function chimeras); C, GLUT4/GLUT1 chimeras with GLUT1 backbone (gain-of-function chimeras).

The abbreviations used are: GSC, GLUT4 storage compartment; EGFP, enhanced green fluorescent protein.
Differentiated 3T3L1 adipocytes were electroporated with 50 μg of GLUT1-EGFP (A) or GLUT4-EGFP (B) fusion constructs. Insulin stimulation (100 nM, 30 min) was performed at 3, 6, 9, and 12 h after transfection. The cells were then processed for fluorescent microscopy as described under “Experimental Procedures.” Open circles, basal; solid squares, insulin. Values are presented as percentage (mean ± S.E.) of plasma membrane (PM) ring obtained by counting 50 cells/condition in 3–5 independent experiments.

Described previously (34). Briefly, the adipocytes were put into suspension by mild trypsinization and electroporated with 50 μg of plasmid DNA under low voltage conditions (160 V, 950 microfarads). After electroporation, the cells were seeded on collagen-coated glass coverslips placed in 6-well plates with complete medium to allow them to recover.

**Time Course Experiments**—Differentiated 3T3L1 adipocytes expressing the different EGFP-tagged plasmids were serum-starved in Dulbecco’s modified Eagle’s medium for 2–2.5 h prior to each experiment. The incubation with or without insulin (100 nM) for 30 min was done at the determined time to end the experiments exactly 3, 6, 9, 12, or 24 h after transfection. The cells were quickly fixed with 4% paraformaldehyde for 15 min at room temperature and blocked with a solution containing 1% bovine serum albumin and 5% donkey serum for another 15 min at room temperature. The coverslips were then washed three times with phosphate-buffered saline and mounted in Vectashield medium.

The translocation of the different GLUT constructs to the plasma membrane was analyzed the next day by wide-field fluorescent microscopy. Translocation data in 3T3L1 adipocytes are expressed as a percent (mean ± S.E.) of positive cells, those showing a plasma membrane ring, obtained by counting 50 cells/condition in 2–5 independent experiments, as described previously (33).

**RESULTS**

**Time-dependent Acquisition of Insulin-stimulated GLUT4 Translocation**—It is well established that the steady-state distribution of GLUT1 in unstimulated adipocytes is primarily plasma membrane, whereas GLUT4 is predominantly confined to intracellular compartments (5, 6, 8). To compare the time-dependent acquisition of insulin-stimulated translocation for newly synthesized GLUT4 and GLUT1 proteins, 3T3L1 adipocytes were transfected with the GLUT4-EGFP or GLUT1-EGFP cDNAs (Fig. 2). As observed previously (33), 3 h following expression, a large fraction of GLUT1 was already localized to the plasma membrane and slowly increased over the next 9 h (Fig. 2A). As expected, there was little, if any, acute (30 min) insulin stimulation of GLUT1 translocation. In contrast, the newly synthesized GLUT4 protein remained intracellular in the basal state with no evidence for any significant plasma membrane accumulation over the 12-h time course examined (Fig. 2B). Although insulin stimulation had no significant effect 3 h after transfection, at 6 h, the newly synthesized GLUT4 protein began to display detectable insulin responsiveness. The extent of insulin-stimulated GLUT4 translocation progressively increased over the next 6 h. These data are in agreement with previous data demonstrating that the newly synthesized GLUT4 protein requires 6–9 h to appropriately sort through the secretory membrane system and accumulate in the GSC without transiting the plasma membrane (33).

**Substitution of Either the GLUT4 Amino-terminal or the Large Intracellular Loop Domains with GLUT1 Inhibits Intracellular Retention**—The most divergent amino acid regions between GLUT4, GLUT1, and other isoforms of the GLUT family of transporters are the amino and carboxyl termini and the large intracellular loop between transmembrane domains 6 and 7. To determine the domains responsible for the distinct subcellular targeting of GLUT4 and GLUT1, we first generated a set of loss-of-function mutants by replacement of the corresponding domains of GLUT4 with the equivalent regions of GLUT1 (Fig. 1B). Substitution of the GLUT4 amino-terminal domain (4–144) or cytoplasmic loop (4–414) with GLUT1 resulted in a loss of basal state retention and almost no significant insulin-stimulated translocation of these chimera proteins (Fig. 3A and B). The relatively rapid default to the plasma membrane, however, was not as fast as that observed for wild type GLUT1. In contrast, substitution of the GLUT4 carboxyl-terminal domain with GLUT1-(4–441) had no significant effect on the basal and time-dependent acquisition of insulin-responsive GLUT4 translocation (Fig. 3C). The importance of the amino-terminal and cytoplasmic loop domains was further confirmed by expression of a double substitution mutant (4–114). This chimera demonstrated a relatively rapid default to the plasma membrane without any significant insulin-stimulated translocation at any of the time points examined (Fig. 3D). These data demonstrate that the carboxyl-terminal domain of GLUT4 does not carry any information required for the initial trafficking of GLUT4 from the secretory membrane transport system to the GSC or insulin-stimulated exit from this compartment. Furthermore, since the double substitution mutant has trafficking characteristics more similar to GLUT1 than either of the single domain substitutions alone, this suggests that both of these domains contribute to this function.

**Substitution of Both the GLUT1 Amino-terminal and the Large Intracellular Loop Domains with GLUT4 Is Sufficient to Recapitulate Normal GLUT4 Trafficking**—One caveat associated with the loss-of-function analysis determined in Fig. 3 is that these domain swaps could result in an aberrantly folded or assembled protein rather than reflecting the requirement of...
these domains. To address this, we prepared a series of gain-of-function chimeras in which these domains of GLUT4 were substituted into the GLUT1 protein (Fig. 1). As observed for the amino-terminal domain loss-of-function mutant, substitution of the GLUT4 amino terminus into GLUT1-(1–411) resulted in a partial, but relatively small, rescue of the GLUT4 phenotype (Fig. 4). That is, the rate of plasma membrane appearance was not as fast as wild type GLUT1, and there was a small degree of insulin-stimulated translocation between 6 and 12 h following new synthesis. Substitution of the GLUT4 cytoplasmic loop (1–141) or the carboxyl-terminal domain (1–114) resulted in a trafficking pattern not significantly different from the wild type GLUT1 protein (Fig. 4, B and C). These data suggest that each of these single domains alone is not sufficient to recapitulate the initial trafficking properties of wild type GLUT4.

Next, we examined the effect of dual GLUT4 domain substitutions into the GLUT1 backbone (Fig. 5). Substitution of the large cytoplasmic loop and carboxyl-terminal GLUT4 domains into GLUT1-(1–144) resulted in a chimera protein that still rapidly defaulted to the plasma membrane but did display a weak insulin-stimulated translocation (Fig. 5). Replacement of GLUT1 with the amino-terminal and carboxyl-terminal GLUT4 domains (1–414) partially resembled wild type GLUT4 but with the presence of a high basal default transport to the plasma membrane (Fig. 5B). In contrast, substitution of the
The large intracellular GLUT4 loop (amino acids 229–271) functionally cooperates with the amino-terminal domain. A, schematic representation of the variants of chimera 1–441 (chimeric loops named 4A to 4C). Dark grey, GLUT4; pale grey, GLUT1. To simplify, the numbers of the amino acids indicated in the chimeras correspond only to GLUT4. B–D, differentiated 3T3L1 adipocytes were electroporated with 50 μg of chimeras 1–44A1 (B), 1–44B1 (C), and 1–44C1 (D). Insulin stimulation (100 nm, 30 min) was performed at 3, 6, 9, and 12 h after transfection. The cells were then processed for fluorescent microscopy as described under “Experimental Procedures.” Open circles, basal; solid squares, insulin. Values are presented as percentage (mean ± S.E.) of cells showing a plasma membrane (PM) ring obtained by counting 50 cells/condition in 3–5 independent experiments.

The FQQI Motif within the Amino-terminal Domain of GLUT4 Is the Major Determinant for Its Intracellular Localization—To further define the amino acids responsible for the regulation of GLUT4 trafficking during initial sorting events, we generated alanine-scanning mutants across the amino-terminal domain of GLUT4. Fig. 6 shows an alignment of the amino terminus of wild type GLUT4 and the 11 alanine mutants generated. The alanine mutants are named with the indication of the two amino acid substitutions performed in each case. As a first approach, we analyzed the percentage of translocation of these 11 mutants at 24 h after transfection to determine the amount of basal and insulin-stimulated translocation under steady-state conditions (data not shown). In this analysis, mutants G4A/F5A, I18A/G19A, and to a lesser extent, Q6A/Q7A and D12A/G13A, showed a higher percentage of translocated cells in the basal state. Based upon these data, we examined the time dependence of basal and insulin-stimulated plasma membrane localization for several selected point mutants (Fig. 7). The G4A/F5A and I18A/G19A mutants had a high rate of basal transport to the plasma membrane essentially identical to that observed for the GLUT1 amino-terminal domain substitution (4–144) in GLUT4 (Fig. 7, A and B). Unexpectedly, the behavior of the Q6A/Q7A mutant was essentially identical to wild type GLUT4 during this early time course (Fig. 7C). These data indicate that Phe⁶ and Ile⁸ are the critical residues within the FQQI motif, whereas the diglutamine residues are dispensable. Although other mutations across the amino terminus had no significant effect on the initial properties of GLUT4 trafficking, the D12A/G13A mutant had a small but significant increase in basal trafficking to the plasma membrane (Fig. 7D). Together these data demonstrate that the key residues in the GLUT4 amino terminus responsible for the appropriate initial sorting into and exit from the GSC are Phe⁶, Ile⁸, and to a lesser extent, Asp¹² and Gly¹³.

The Large Intracellular GLUT4 Loop (Amino Acids 229–271) Functionally Cooperates with the Amino Terminus—To further examine the regions of the large intracellular loop between transmembrane domains 6 and 7, we generated another set of gain-of-function mutants as variants of chimera 1–441. In these chimeras, the amino terminus of GLUT4 and three different regions of the cytoplasmic loop (named 4A to 4C) were replaced into GLUT1 (Fig. 8A). Substitution of the amino terminus of GLUT4 plus the first half (1–44A1) or the middle region (1–44B1) of the GLUT4 loop into GLUT1 partially recapitulated normal GLUT4 trafficking but with a slightly higher level of plasma membrane localization in the basal state (Fig. 8, B and C). In contrast, substitution of the second half of the loop together with the amino-terminal domain of GLUT4 into GLUT1 (1–44C1) did not display any significant difference when compared with the 1–411 chimera (Fig. 8D). These data suggest that amino acids in the third part of the intracellular loop of GLUT4 are not required for the appropriate initial trafficking of GLUT4 and retention into the GSC. Taken together, these data demonstrate that the amino terminus plus the continuous sequence of the large intracellular loop (amino acids 229–271) are required to recapitulate newly synthesized GLUT4 trafficking.

**DISCUSSION**

It is well known that in muscle and adipose tissue, the insulin-sensitive glucose transporter GLUT4 slowly recycles between the plasma membrane and various intracellular compartments with the steady-state levels at the cell surface determined by the relative rates of exocytosis and endocytosis (5, 10, 11, 18, 35). Insulin stimulation results in a marked enhancement in the rate of exocytosis with a small, if any, decrease in the rate of endocytosis. Although a few studies have specifically examined the endocytosis and recycling of GLUT4,
(15, 29, 36), most have typically relied on steady-state distributions to identify functional motifs responsible for GLUT4 trafficking events (19–28, 31). The interpretation of these data is difficult as an alteration of one trafficking step can appear to affect a different step that is kinetically linked. Moreover, specific structural motifs may be required for unique trafficking steps, whereas other motifs may be required at multiple sorting steps, providing overlapping function. Despite these caveats, it is generally accepted that both the amino-terminal FQIQI motif and the carboxyl-terminal SLL motif are necessary for efficient plasma membrane endocytosis (27, 28, 36). Recently, we have observed that newly synthesized GLUT4 protein traffics through the secretory membrane system (endoplasmic reticulum and the Golgi apparatus) and is then directly sorted to the specialized GLUT4 storage compartments without having to transit the plasma membrane to acquire insulin responsiveness (33). In the basal state, there is a slow exit of GLUT4 from the GSC and subsequent traffic to the plasma membrane followed by endocytosis and recycling back to the GSC. These data demonstrated that time-dependent acquisition of insulin responsiveness correlates with the entry of the newly synthesized GLUT4 protein into the GSC. Based upon these data, we took advantage of the temporal separation of these events to examine the GLUT4 motifs responsible for the initial sorting of GLUT4 into and out of the GSC in 3T3L1 adipocytes.

Specifically, we have found that the newly synthesized GLUT4 protein rapidly exits the secretory membrane system and accumulates at the plasma membrane in an insulin-independent manner. In contrast, GLUT4 accumulates in intracellular compartments without any appreciable concentration at the cell surface. Although insulin does induce a robust translocation of GLUT4 to the plasma membrane, this process takes 6–9 h following new synthesis and is consistent with a slow sorting step from the Golgi to the GSC (33). Analysis of the time-dependent trafficking of GLUT4/GLUT1 loss-of-function chimeras demonstrated that both the amino terminus and the large cytoplasmic loop between transmembrane domains 6 and 7 are necessary for the appropriate sorting and retention of the newly synthesized GLUT4 protein into the GSC. However, replacement of the carboxyl-terminal GLUT4 domain with GLUT1 had no significant effect. Previous studies have demonstrated that the carboxyl-terminal domain, particularly the SLL and TELEYLGPS motifs, is an important element in endocytosis and recycling to the GSC (15, 31, 32). Thus, our data demonstrate that these motifs do not play a significant role in the initial entry into and exit from the GSC but are still consistent with a requirement for appropriate plasma membrane endocytosis and subsequent recycling back to the GSC.

Gain-of-function chimeras, on the other hand, demonstrated that none of the individual cytoplasmic domains alone were able to fully recapitulate the sorting and insulin-stimulated translocation of GLUT4. However, substitution of the GLUT4 amino terminus plus the large cytoplasmic loop generated a chimera that was functionally indistinguishable from the wild type GLUT4. These data directly demonstrate that GLUT4 contains multiple non-contiguous elements that either fold into a specific functional domain or direct several sorting events between the Golgi and the GSC, each utilizing distinct motifs. In either case, it is clear that the normal pattern of GLUT4 trafficking is a complex, multistep process with distinct sequence requirements for different steps in the transport process.

Another important aspect of the present data is the analysis of newly synthesized GLUT4 trafficking when compared with previous studies using steady-state expression to identify GLUT4 trafficking motifs. Previous steady-state measurements have resulted in divergent conclusions and can now be reconciled depending on the specific assay system employed. For example, it was originally reported that the amino-terminal domain was necessary for GLUT4 intracellular sequestration, whereas subsequent studies found no evidence for the amino terminus but pointed to the carboxyl terminus. Consistent with our data, the analysis implicating the amino-terminal domain was performed using a viral expression system over a short period of time (6 h) (19, 20). In contrast, studies implicating the carboxyl terminus either utilized steady-state expression or labeled only exocytically exposed GLUT4 (21–23). Thus, the former studies were probably examining the initial sorting of the newly synthesized GLUT4 protein to the GSC, whereas the latter were primarily analyzing plasma membrane endocytosis and intracellular sorting back to the GSC. It has to be noted that these domains may also functionally overlap or supply redundant functions as the amino-terminal domain also plays a role in plasma membrane endocytosis (27, 36). Furthermore, in agreement with our data, the transient transfection of primary adipocytes demonstrated that 4 h after transfection, the trafficking of the dileucine mutant (LL489/490AA) was identical to wild type GLUT4, whereas the amino-terminal F5A mutant had marked elevation of basal cell surface levels (36).

Within the amino terminus, the FQIQI motif has received the most attention, and in steady-state measurements, mutation of this domain results in plasma membrane accumulation (19, 25). These data are also consistent with our findings that the 44A/F5A and ISA/G9A point mutants have a faster exit to the plasma membrane. Since these mutants also have a reduced rate of endocytosis, this contributes to the observed steady-state increase at the cell surface. We have also demonstrated for the first time that the large intracellular loop of GLUT4 is necessary to completely recapitulate initial GLUT4 trafficking. Although we have not yet mapped specific sites in the cytoplasmic loop, our data indicate that the region between amino acids 229–271 may be required, possibly at different steps of the sorting process of the newly synthesized GLUT4 between the Golgi and the GSC. Recently, it has been reported that TUG ( tether, containing a UBX domain, for GLUT4) is a tethering protein responsible for intracellular sequestration of GLUT4 (37). The apparent binding site for TUG in GLUT4 is amino acids 263–273, which is partially included in the chimera 1–44B1 that partially displays restoration of wild type GLUT4 sorting. However, since the substitution of the amino terminus of GLUT4 and the second half of the loop into GLUT1 was not sufficient to completely recapitulate normal GLUT4 trafficking, the contribution of this or other non-contiguous motifs within the cytoplasmic loop of GLUT4 to this function needs further investigation.

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