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The Glucose Transporter 4 FQQI Motif Is Necessary for Akt Substrate of 160-Kilodalton-Dependent Plasma Membrane Translocation But Not Golgi-Localized γ-Ear-Containing Arf-Binding Protein-Dependent Entry into the Insulin-Responsive Storage Compartment

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Newly synthesized glucose transporter 4 (GLUT4) enters into the insulin-responsive storage compartment in a process that is Golgi-localized γ-ear-containing Arf-binding protein (GGA) dependent, whereas insulin-stimulated translocation is regulated by Akt substrate of 160 kDa (AS160). In the present study, using a variety of GLUT4/GLUT1 chimeras, we have analyzed the specific motifs of GLUT4 that are important for GGA and AS160 regulation of GLUT4 trafficking. Substitution of the amino terminus and the large intracellular loop of GLUT4 into GLUT1 (chimera 1-441) fully recapitulated the basal state retention, insulin-stimulated translocation, and GGA and AS160 sensitivity of wild-type GLUT4 (GLUT4-WT). GLUT4 point mutation (GLUT4-F5A) resulted in loss of GLUT4 intracellular retention in the basal state when coexpressed with both wild-type GGA and AS160. Nevertheless, similar to GLUT4-WT, the insulin-stimulated plasma membrane localization of GLUT4-F5A was significantly inhibited by coexpression of dominant-interfering GGA. In addition, coexpression with a dominant-interfering AS160 (AS160-4P) abolished insulin-stimulated GLUT4-WT but not GLUT4-F5A translocation. GLUT4 endocytosis and intracellular sequestration also required both the amino terminus and large cytoplasmic loop of GLUT4. Furthermore, both the FQQI and the SLL motifs participate in the initial endocytosis from the plasma membrane; however, once internalized, unlike the FQQI motif, the SLL motif is not responsible for intracellular recycling of GLUT4 back to the specialized compartment. Together, we have demonstrated that the FQQI motif within the amino terminus of GLUT4 is essential for GLUT4 endocytosis and AS160-dependent intracellular retention but not for the GGA-dependent sorting of GLUT4 into the insulin-responsive storage compartment. (*Molecular Endocrinology* 21: 3087–3099, 2007)

THE FACILITATIVE glucose transporter (GLUT) proteins are a large family of 12 membrane-spanning domain proteins with relative degrees of specificity for monosaccharides with distinct but overlapping distribution and kinetic properties (reviewed in Refs. 1 and 2). The major insulin-responsive GLUT4 is primarily expressed in striated muscle and adipose tissue and is responsible for the postprandial disposal of glucose from the circulation (3–5). In the basal state, GLUT4 undergoes a slow but continuous recycling between the plasma membrane and several intracellular compartments, such that approximately only 5% of the total GLUT4 protein pool is within the plasma membrane. In response to acute insulin stimulation, however, the rate of GLUT4 exocytosis markedly increases, whereas also a small decrease in endocytosis occurs, so that a new steady-state distribution is reached with approximately 50% of the GLUT4 protein now localized in the cell surface (3, 6, 7). After the removal of the insulin signal, the rate of exocytosis subsequently decreases, and this trafficking system reverts back to the basal steady-state distribution.

Based upon the importance of this specific trafficking system to maintain normal glucose homeostasis, numerous studies have attempted to identify the specific *cis*-elements and *trans*-factors responsible for the dynamic regulatory properties of GLUT4. Despite this intensive effort, substantial discrepancy remains with regard to the specific structural motifs responsible for the regulation of GLUT4 trafficking (reviewed in Refs.

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Abbreviations: AS160, Akt substrate of 160 kDa; AS160–4P, dominant-interfering AS160 mutant; EGFP, enhanced GFP; GAP, GTPase-activating protein; GFP, green fluorescent protein; GGA, Golgi-localized γ-ear-containing Arf-binding protein; GGA-DN, dominant-interfering GGA mutant; GLUT, glucose transporter; IRC, insulin-responsive compartment; WT, wild type.

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5, 6, and 8). Several studies have implicated a requirement for the SLL motif and the acidic cluster within the carboxyl-terminal domain for both GLUT4 endocytosis and recycling back to the insulin-responsive storage compartment (IRC) (9–11). (In this manuscript, the terms internalization and endocytosis refer to the GLUT4 molecules that enter into the cell from the plasma membrane. The term recycling refers to the GLUT4 molecules that after internalization traffic back to the IRC and have the ability to respond to insulin.) In contrast, others have indicated that the GLUT4 amino-terminal FQQI motif is required, whereas some other studies have suggested that a combination of several motifs is necessary (12–16). In addition to the confusing literature on the required GLUT4 *cis*-elements, a variety of *trans*-factors have also been implicated in the regulated trafficking of GLUT4 (reviewed in Refs. 5, 17, and 18). However, recent studies have provided compelling evidence for a novel Akt/protein kinase B substrate, termed Akt Substrate of 160 kDa (AS160), that suppresses the basal state translocation of GLUT4 to the cell surface (19, 20). AS160 functions as a Rab GTPase-activity protein (GAP) inhibiting a putative Rab protein. *In vitro*, AS160 displays activity toward Rabs 2A, 8A, 10, and 14 (21). Recently, Rab10 has been reported to function as an AS160 substrate necessary for insulin-stimulated GLUT4 translocation whereas another report implicated Rab8A and Rab14 (22, 23). In any case, AS160 was also reported to associate with another GLUT4 vesicle-associated protein, the insulin-responsive aminopeptidase in 3T3L1 adipocytes in the basal state, and to dissociate from it upon insulin stimulation (24). In addition, the insulin-stimulated Akt-dependent phosphorylation of AS160 was hypothesized to inhibit its intrinsic GAP activity, thereby allowing the exit of GLUT4 from its intracellular sequestration to the plasma membrane (19, 25). Thus, it has been postulated that AS160 is responsible for the regulated trafficking of GLUT4 by controlling the dynamic retention within and exit from the IRC.

In addition to the regulated recycling of the preexisting GLUT4 population by AS160, adipocytes appear to regulate the trafficking of the newly synthesized GLUT4 protein through the Golgi complex *en route* to the IRC (26, 27). In this regard, after the initial biosynthesis, GLUT4 undergoes a relatively slow sorting process (6–9 h) from the *trans*-Golgi network to the IRC that is dependent upon the Golgi-localized γ-ear-containing Arf-binding protein (GGA) adaptor protein (27). GGA is a monomeric/multidomain coat protein shown to be involved in the intracellular trafficking of several cargo molecules. Although the cargo-binding domain of GGA (VHS) does not appear to directly associate with GLUT4, there are several other well-established cargo proteins that contain VHS consensus binding motifs (DxxLL) that have been shown to directly interact with GGA. In particular, sortilin has recently been shown to play an important role in the formation of the specialized GLUT4 IRC in adipocytes (28). In support of a critical role for sortilin in the sorting of GLUT4, chemical cross-linking and yeast two-hybrid studies indicated an interaction of the luminal domains of GLUT4 and insulin-responsive aminopeptidase with sortilin (29). Together, these data suggest a model in which sortilin serves as the cargo adaptor protein linking GLUT4 to GGA-coated transport vesicles that are then sorted to the IRC.

The identification of GGA as a regulator of GLUT4 biosynthetic entry into the IRC and AS160 as a possible regulator of GLUT4 exit from the IRC has provided specific reagents to dissect and resolve the structural motifs of GLUT4 that are directly or indirectly responsible for these distinct trafficking steps. Thus, to resolve some of the discrepancies associated with the functional motifs within GLUT4, we have examined the relative effects of various GLUT4/GLUT1 chimeras and GLUT4 point mutations on AS160 and GGA sensitivity. These data demonstrate that both the amino terminus and the cytoplasmic loop of GLUT4 are required for the GGA-dependent biosynthetic entry of GLUT4 into the IRC. Moreover, the amino-terminal FQQI motif is not only essential for GLUT4 endocytosis but is also required for AS160-dependent intracellular retention in the IRC.

RESULTS

3T3L1 Adipocytes as a System to Study GLUT4 Trafficking

Over the past decade, insulin-regulated GLUT4 trafficking has been extensively studied using the well-established cultured 3T3L1 adipocytes as a model system of adipocytes *in vivo*. In the present study, we have taken advantage of a variety of GLUT4/GLUT1 chimera proteins to investigate the structural motifs of GLUT4 that are important for GGA- and AS160-regulated GLUT4 trafficking. A schematic representation of the different GLUT4/GLUT1 chimera constructs used in this study is shown in Fig. 1.

To compare the relative expression levels of the different sets of cotransfected proteins, we electroporated 50 μg wild-type GLUT4 (GLUT4-WT) or various chimera proteins with wild-type GGA (GGA-WT), a dominant-interfering GGA mutant (GGA-DN) that consists of the VHS-GAT domains of GGA1, wild-type AS160 (AS160-WT), or a dominant-interfering AS160 mutant (AS160-4P) in which four of the Akt phosphorylation sites are mutated to alanine. Eighteen hours later, cells were treated with or without insulin and analyzed by both fluorescent microscopy and immunoblotting. The percentage of cells coexpressing both plasmids was 90% or more in all cases. Figure 2, A and B, displays representative confocal microscopy images showing similar expression levels of both GLUT4 and GGA (Fig. 2A, panels a–f for GGA-WT and panels g–l for GGA-DN, respectively) or AS160 (Fig. 2B, panels a–f for AS160-WT and panels g–l for AS160-4P, respectively) in 3T3L1 adipocytes. All the

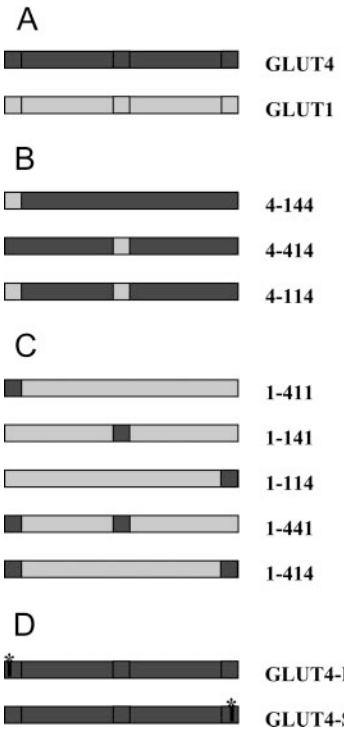

Fig. 1. Schematic Representation of the Main GLUT4/GLUT1 Chimera Constructs and Point Mutations Used in this Study

A, Wild-type constructs; B, chimeras backbone GLUT4; C, chimeras backbone GLUT1; and D, GLUT4 point mutations. GLUT4 is represented in *dark gray* and GLUT1 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 (except in chimera 4-144, which 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 carboxy terminus. The nomenclature of the chimeras is described in *Materials and Methods*. The *asterisks* represent point mutations.

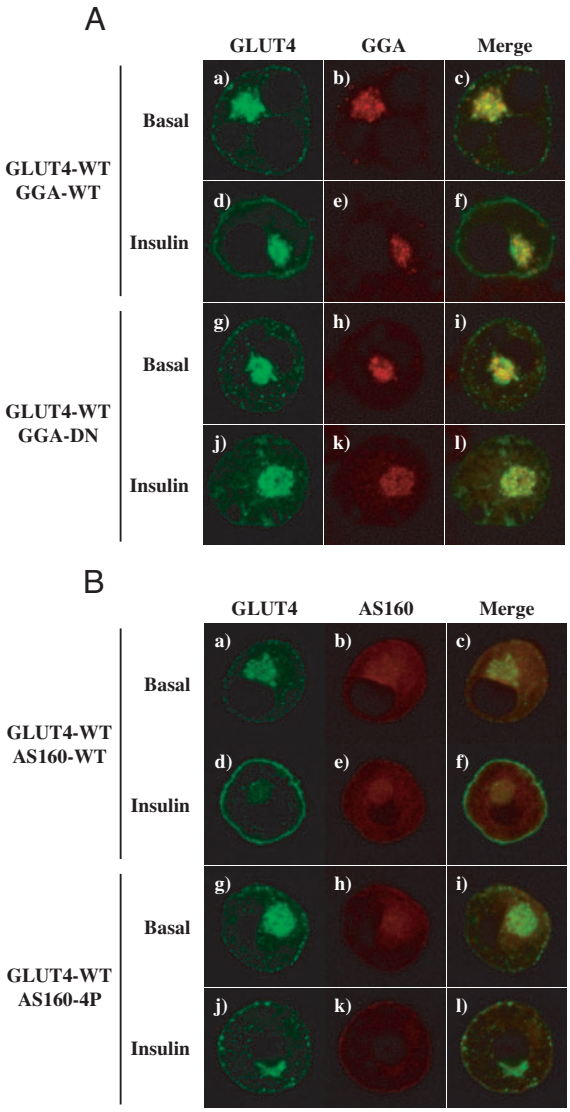

Fig. 2. Comparison of Coexpression Levels of Various GLUT4 Constructs with Either GGA or AS160

A, Representative confocal-microscopy images of cells coexpressing wild-type GLUT4-EGFP with DsRed-GGA-WT or DsRed-GGA-DN in basal (a–c and g–i, respectively) or insulin-stimulated conditions (100 nM for 30 min; d–f and j–l, respectively); B, representative confocal-microscopy images of cells coexpressing wild-type GLUT4-EGFP with Flag-AS160-WT or Flag-AS160-4P in basal (a–c and g–i, respectively) or insulin-stimulated conditions (100 nM for 30 min; d–f and j–l, respectively).

images were taken by Zeiss LSM510 microscope under the same scanning settings and laser power.

We further quantified the relative expression of GLUT4 proteins in the presence of the different GGA and AS160 constructs by Western blot analysis. Representative blots are shown in Fig. 3A for cells coexpressing GLUT4 and GGA and in Fig. 3B for cells coexpressing GLUT4 and AS160. The expression of each one of the GLUT4 reporter constructs was detected by using an anti-green fluorescent protein (anti-GFP) antibody (Fig. 3A, *upper panel*). Due to the absence of the myc3 tag in 4-144 and 4-414 chimeras, they migrate with a lower molecular weight band in comparison with GLUT4-WT and GLUT4-F5A. In any case, the expression levels of the GLUT4 reporters were similar as was the expression of GGA-WT (Fig. 3A, *left middle panel*). However, we were not able to detect GGA-DN because the epitope of the GGA antibody recognizes only part of the hinge-GAE domain of GGA, which is missing in the GGA-DN construct. Therefore, we used HA-GGA-DN to compare the expression level between the different GLUT4 constructs (Fig. 3A, *right middle panel*). To make sure that when coexpressed, HA-GGA-DN functioned as a dominant-interfering mutant similar to the DsRed-GGA-DN, we analyzed on each case the levels of GLUT4 plasma membrane localization (data not shown). Both of these tagged mutant GGA proteins inhibited GLUT4-WT translocation to the same extent as previously reported (27). In any case, expression of both the GLUT4

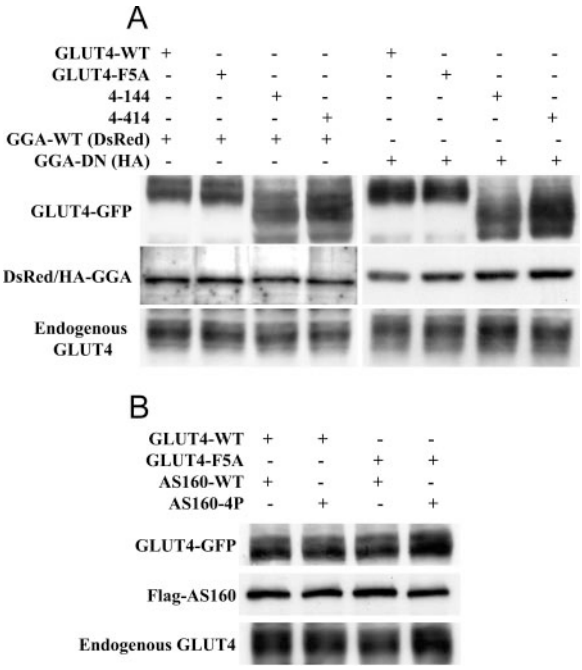

Fig. 3. Comparison of Protein Expression Levels by Immunoblotting

A, Representative immunoblots are shown from two independent experiments of cells coexpressing GLUT4-WT, GLUT4-F5A, and chimeras 4-144 and 4-141 EGFP constructs with DsRed-GGA-WT or HA-GGA-DN; B, representative immunoblots are shown from two independent experiments of cells coexpressing GLUT4-WT and GLUT4-F5A EGFP constructs with Flag-AS160-WT or Flag-AS160-4P. Western blotting was performed as described in *Materials and Methods* using rabbit polyclonal anti-GFP for the GLUT4 constructs, mouse monoclonal M2 anti-Flag for the AS160 constructs, custom-made rabbit polyclonal anti-GGA1 for the GGA-WT construct, mouse monoclonal anti-HA for the GGA-DN, and carboxyl-terminal IAO2 antibody for endogenous GLUT4 as primary antibodies.

reporters and GGA proteins had no significant effect on the expression of the endogenous GLUT4 protein detected using the carboxyl-terminal IAO2 antibody (Fig. 3A, *bottom panel*). Similarly, cotransfection with the expression plasmids for GLUT4 and AS160 demonstrated that both GLUT4-WT and GLUT4-F5A were expressed at essentially identical levels (Fig. 3B, *upper panel*), as were the AS160-WT and AS160-4P mutant (Fig. 3B, *middle panel*). The *lowest panel* represents endogenous GLUT4 that again remained unaffected (Fig. 3B, *lower panel*).

The Amino Terminus and the Large Intracellular Loop of GLUT4 Are Both Necessary and Sufficient for GGA-Dependent Entry of GLUT4 into the IRC

We have reported previously that entry of newly synthesized GLUT4 into the IRC is GGA dependent, whereas GLUT1 trafficking to the plasma membrane after biosynthesis is not affected by the expression of a dominant-interfering GGA mutant (27). These data suggest that although GLUT4 does not contain the consensus DxxLL motif required for GGA cargo binding, there might be some distinct sequence in GLUT4 different from GLUT1 that may regulate this trafficking step. Therefore, to investigate the regions of GLUT4 that may be involved in GGA sensitivity, we used GLUT4/GLUT1 chimera proteins in which the three major intracellular domains of the GLUT proteins were exchanged (Fig. 1). As previously observed (27, 30, 31), coexpression of GLUT4-WT with GGA-WT had no significant effect on the basal or insulin-stimulated GLUT4 translocation as compared with GLUT4-WT alone (Fig. 4). However, coexpression of GLUT4-WT with GGA-DN appeared to reduce the basal and significantly inhibited the insulin-stimulated GLUT4 plasma membrane localization, although the fold insulin stimulation was similar to the coexpression with GGA-WT. This probably results from a small amount of newly synthesized GLUT4 protein that can enter the IRC in the presence of GGA-DN and, once in the IRC, is fully capable of undergoing insulin-stimulated translocation, as previously observed for the insulin-responsive aminopeptidase (32).

In addition, Fig. 4 also shows the results of a series of GLUT4/GLUT1 loss-of-function chimeras in the presence of GGA-WT or GGA-DN. Substitution of the amino terminus or the cytoplasmic loop of GLUT4 for GLUT1 (4-144 and 4-414, respectively) resulted in loss of basal state intracellular retention, but these chimeras still showed significant insulin-stimulated translocation when cotransfected with GGA-WT. Neverthe-

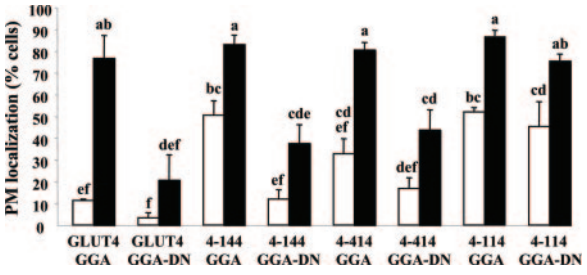

Fig. 4. Effect of Dominant-Interfering GGA on the Basal and Insulin-Stimulated Translocation of Various Loss-of-Function GLUT4/GLUT1 Chimera Mutants

Differentiated 3T3L1 adipocytes were cotransfected with 50 μg each of a GLUT4/GLUT1-EGFP chimera construct and DsRed-GGA-WT or DsRed-GGA-DN mutant. After an overnight incubation period, the cells were either insulin stimulated (100 nM, 30 min) or left untreated as described in *Materials and Methods*. *White bars*, Basal; *black bars*, insulin. Values are presented as percentage (mean $\pm$ SEM) of cells showing a plasma membrane (PM) ring obtained by counting 100 GGA-positive cells per condition and averaged from three to five independent experiments. Statistical analysis was performed using a one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. *Identical letters* indicate *bars* that are not statistically different from each other ($P < 0.05$).

less, in the presence of GGA-DN, chimeras 4-144 and 4-414 displayed reduced basal plasma membrane localization with a reduction in the total extent of insulin-stimulated translocation when compared with coexpression with GGA-WT. However, because these mutants still display some degree of insulin-stimulated translocation, this result suggests that a portion of these mutants is delivered to the IRC. It is also important to note that in the presence of GGA-DN, the plasma membrane localization of the 4-144 and 4-414 chimeras are not significantly different from each other in either basal or insulin-stimulated conditions. In addition, chimera 4-114, which contains the amino terminus and the intracellular loop of GLUT1 in the GLUT4 backbone, presented high levels of plasma membrane localization in basal and insulin-stimulated states in the presence of either GGA-WT or GGA-DN, suggesting a requirement of both the amino terminus and the intracellular loop of GLUT4 domains for the full extent of GGA sensitivity.

To further assess this result, we next turned to a series of gain-of-function mutants in which various domains of GLUT4 were substituted into the GLUT1 backbone protein. It is well known that during steady-state conditions, the distribution of GLUT1 in adipocytes is primarily at the plasma membrane (33, 34). As shown in Fig. 5A, neither GGA-WT nor GGA-DN had any significant effect on the plasma membrane localization of GLUT1-WT under basal or insulin-stimulated conditions. Moreover, substitution of either one of the GLUT4 domains (amino-terminal, 1-411; large intracellular loop, 1-141; or carboxyl-terminal, 1-114) into GLUT1 could not mimic GLUT4 function, and these constructs were completely refractory to the presence of GGA-WT or GGA-DN. The only exception was chimera 1-411, which when coexpressed with GGA-DN, displayed a small but statistically significant lower basal level of plasma membrane localization, suggesting that the amino-terminal domain of GLUT4 may have a partial role in GGA sensitivity (Fig. 5A). Similarly, the double substitution of the amino- and carboxyl-terminal GLUT4 domains into GLUT1 (1-414) also displayed a relatively high basal plasma membrane distribution that was significantly reduced by GGA-DN in comparison with GGA-WT but not at the level of inhibition observed with GLUT4-WT (Fig. 5B). This result indicates that chimera 1-414 containing the GLUT4 amino terminus partially recovers the GGA sensitivity. In contrast, substitution of the amino terminus and large intracellular loop of GLUT4 into GLUT1 (1-441) fully recapitulated the basal state retention and insulin-stimulated GLUT4 translocation in the presence of GGA-WT, and more importantly, this chimera completely restored GGA sensitivity when coexpressed with GGA-DN (Fig. 5B). Thus, the amino terminus and the large cytoplasmic loop of GLUT4 are necessary and sufficient to recapitulate the appropriate GGA-dependent sorting of GLUT4 into the IRC. In the absence of these sequences, GLUT4 is therefore missorted into compartments that are not GGA sensitive.

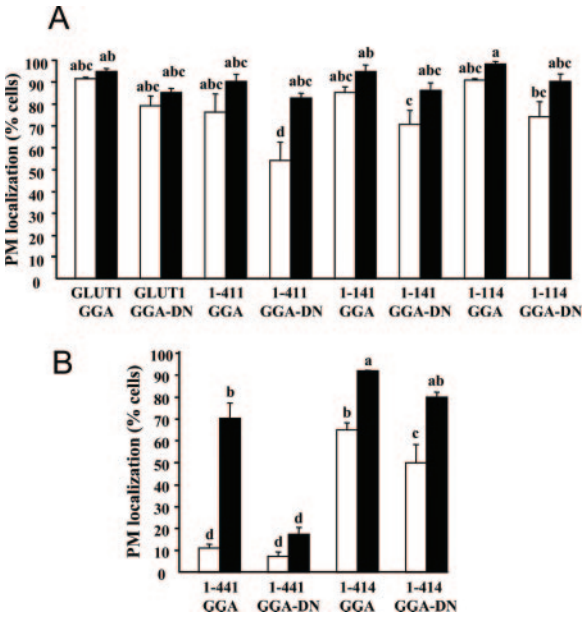

Fig. 5. GGA Sensitivity of Various Gain-of-Function GLUT4/GLUT1 Chimera Mutants

Differentiated 3T3L1 adipocytes were cotransfected with 50 μg each of single-domain GLUT4/GLUT1-EGFP chimera constructs (A) or double-domain GLUT4/GLUT1-EGFP chimera constructs (B) as indicated. These chimera cDNAs were cotransfected with either DsRed-GGA-WT or DsRed-GGA-DN mutant. After an overnight incubation period, the cells were either insulin stimulated (100 nM for 30 min) or left untreated as described in *Materials and Methods*. *White bars*, basal; *black bars*, insulin. Values are presented as percentage (mean $\pm$ SEM) of cells showing a plasma membrane (PM) ring obtained by counting 100 GGA-positive cells per condition and averaged from three to six independent experiments. Statistical analysis was performed using a one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. *Identical letters* indicate *bars* that are not statistically different from each other ($P < 0.05$).

The Amino Terminus and the Large Intracellular Loop of GLUT4 Are Both Necessary and Sufficient for AS160-Dependent Exit of GLUT4 from the IRC

As described above, the Rab GAP protein AS160 has been recently reported to play an important functional role in the regulation of GLUT4 trafficking (19, 23, 25). This protein is a substrate for Akt and contains six sites of phosphorylation. Mutation to alanine of two or more of these sites has been demonstrated to markedly inhibit insulin-stimulated GLUT4 translocation in adipocytes (19, 20). To investigate the GLUT4 motifs involved in the direct or indirect interaction with AS160, we analyzed the extent of insulin-stimulated translocation of different GLUT4/GLUT1 chimera proteins when coexpressed together with AS160-WT or the dominant-interfering mutant AS160-4P (Fig. 6). GLUT1-WT and the GLUT4/GLUT1-enhanced GFP (EGFP) gain-of-function chimeras containing a single

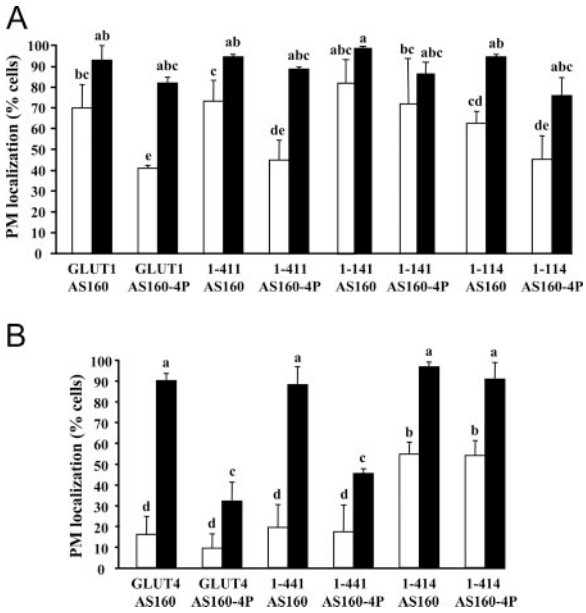

Fig. 6. AS160 Sensitivity of Various Gain-of-Function GLUT4/GLUT1 Chimera Mutants

Differentiated 3T3L1 adipocytes were cotransfected with 50 μg each of single-domain GLUT4/GLUT1-EGFP chimera constructs (A) or double-domain GLUT4/GLUT1-EGFP chimera constructs (B) as indicated. These chimera cDNAs were cotransfected with either Flag-AS160-WT or Flag-AS160-4P mutant. After an overnight incubation period, the cells were either insulin stimulated (100 nM for 30 min) or left untreated as described in *Materials and Methods*. *White bars*, basal; *black bars*, insulin. Values are presented as percentage (mean ± SEM) of cells showing a plasma membrane (PM) ring obtained by counting 100 AS160-positive cells per condition and averaged from three independent experiments. Statistical analysis was performed using a one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. Identical letters indicate bars that are not statistically different from each other ($P < 0.05$).

GLUT4 domain in a GLUT1 backbone protein displayed relatively high basal plasma membrane localization in the presence of both AS160-WT and AS160-4P when compared with GLUT4-WT, although we did observe a statistically significant decrease in basal translocation of GLUT1 and chimera 1-411 in the presence of AS160-4P in comparison with AS160-WT (Fig. 6A). Nevertheless, no significant differences were found in the insulin-stimulated translocation levels in any of the constructs when coexpressed either with AS160-WT or AS160-4P.

On the other hand, when coexpressed with AS160–4P, GLUT4-WT had reduced plasma membrane localization in the basal state and significantly inhibited insulin-stimulated translocation (Fig. 6B). Similar to the results obtained using GGA-DN, the GLUT1 chimera 1-414, with the amino- and carboxyl-terminal domains of GLUT4 substituted into GLUT1, was not significantly different from the single GLUT4 domain substitutions. That is, this chimera displayed a high basal state plasma membrane distribution compared with GLUT4-WT, and neither the basal nor insulin-stimulated translocation was strongly inhibited by AS160-4P (Fig. 6B). However, the 1-441 chimera with the amino-terminal and the cytoplasmic domain of GLUT4 in the GLUT1 backbone protein again fully recapitulated GLUT4-WT behavior when coexpressed with both AS160-WT and AS160-4P (Fig. 6B). Taken together, these data demonstrate that the amino terminus and large cytoplasmic loop of GLUT4 are together both necessary and sufficient to impart GGA- and AS160-dependent GLUT4 basal state retention and insulin-stimulated translocation in 3T3L1 adipocytes.

The Amino Terminus and the Large Intracellular Loop of GLUT4 Are Both Necessary and Sufficient for GLUT4 Internalization

The data presented in Figs. 4–6 demonstrate that the amino terminus and the large intracellular loop of GLUT4 are both necessary and sufficient for the GGA-dependent entry of newly synthesized GLUT4 into the IRC and AS160-dependent exit of GLUT4 from the IRC. Because we had observed that several of the chimeras had high levels of cell surface localization in the basal state, one of the possibilities could be due to a reduced rate of endocytosis concomitant with an increased rate of exocytosis. To address this issue, the exofacial myc epitope of different GLUT4 constructs was labeled with a c-Myc antibody at 4 C, and a time course of c-Myc antibody internalization was determined after warming of the cells to 37 C. Consistent with previous reports, the $t_{1/2}$ for GLUT4-WT endocytosis was approximately 30 min with maximal intracellular redistribution (~90%) occurring by 120 min (Fig. 7A). In contrast, the initial rate of GLUT1 endocytosis was comparatively slower and with a lower steady-state extent of internalization such that only 40% of the surface-labeled GLUT1 was apparently internalized (Fig. 7A). This probably reflects the rapid recycling of GLUT1 back to the cell surface after endocytosis, whereas once internalized, GLUT4 becomes sequestered in the IRC.

Furthermore, substitution of the amino-terminal GLUT4 domain (1-411) or the carboxyl-terminal GLUT4 domain (1-114) into GLUT1 resulted in chimeras that displayed essentially identical endocytosis properties as GLUT1-WT (Fig. 7B). Although substitution of both the amino terminus and the intracellular loop of GLUT4 into GLUT1 (1-441) did not fully restore the initial rate of GLUT4-WT endocytosis, the extent of endocytosis by 120 and 240 min was identical (Fig. 7C). In addition, recycling back to the IRC of chimera 1-441 was confirmed by a second round of stimulation at 4 h, which showed a full response of insulin-stimulated translocation to the plasma membrane that was identical to that of GLUT4-WT (data not shown). Interestingly, the amino- and carboxyl-terminal GLUT4 domains substituted into GLUT1 (1-414) showed similar initial rates of endocytosis as chimera 1-441 but re-

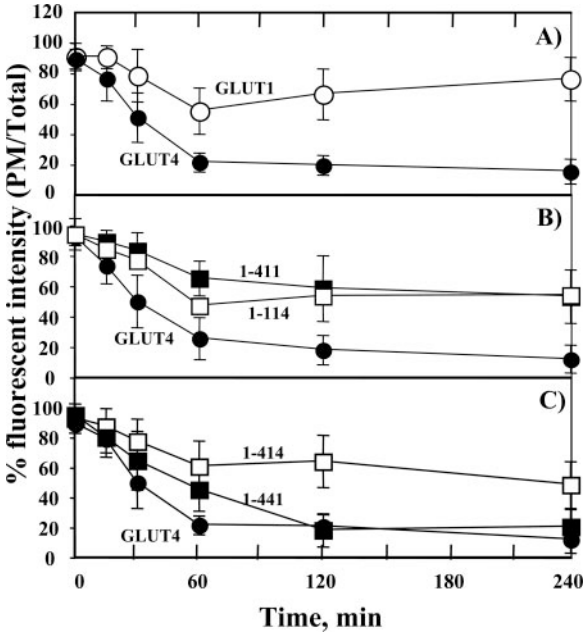

Fig. 7. Determination of Plasma Membrane Endocytosis for Gain-of-Function GLUT4/GLUT1 Chimera Mutants

A, GLUT4-WT (●) and GLUT1-WT (○); B, GLUT4-WT (●), single-domain chimera mutants chimera 1-411 (■) and chimera 1-114 (□); C, GLUT4-WT (●), double-domain chimera mutants chimera 1-441 (■) and chimera 1-414 (□) containing an exofacial myc epitope tag were transfected into 3T3L1 adipocytes. After an overnight incubation, all the cells were insulin stimulated (100 nM for 30 min) to induce the translocation of all the constructs that were intracellularly sequestered. The cells were then cooled to 4 C, and the exofacial myc tag was labeled with the c-Myc antibody for 1 h. The cells were extensively washed to remove the unbound Myc antibody and residual insulin and then warmed to 37 C to initiate endocytosis. The cells were fixed at 0, 15, 30, 60, 120, and 240 min after warming to 37 C and labeled with a Texas Red antimouse secondary antibody. The amount of plasma membrane-localized myc epitope *vs.* total cell myc epitope was quantified as described in *Materials and Methods*. Values are presented as percentage (mean ± SEM) showing the ratio of Texas Red signal intensity in plasma membrane (PM) compared with the entire cell fluorescence. Values are presented as percentage (mean ± SEM) from the quantification of 10 cells per time point averaged from three independent experiments.

sulted in a lower extent of internalization at steady state. These data confirm that the full extent of endocytosis and intracellular sequestration requires only the amino terminus and large cytoplasmic loop of GLUT4, although the carboxyl-terminal domain does appear to play a small contributing role to enhance the rate of initial endocytosis.

The FQQI Motif within the Amino-Terminal Domain of GLUT4 Is Necessary for AS160-Dependent Exit of GLUT4 from the IRC

To further delineate the specific sequences required within these domains to regulate GLUT4 trafficking, we first made a series of overlapping chimeras within the large cytoplasmic loop of GLUT4. However, all of these mutants resulted in partial trafficking defects and GGA- and AS160-sensitive phenotypes that were not sufficiently different from each other (data not shown). Therefore, we next focused our attention to the amino-terminal GLUT4 FQQI motif that has previously been implicated in both insulin-regulated exocytosis and plasma membrane endocytosis. Because previous studies have reported that the carboxyl-terminal di-leucine motif SLL also plays an important role in endocytosis, we examined this motif as well to use it as a comparison in our system. As previously observed, expression of GGA-DN but not GGA-WT with GLUT4-WT resulted in a significant inhibition of insulin-stimulated GLUT4 translocation (Fig. 8A). Coexpression of GGA-WT with a GLUT4 mutant in which the phenylalanine at position 5 was mutated to alanine (GLUT4-F5A) resulted in loss of GLUT4 intracellular retention in the basal state (Fig. 8A). This effect was not due to the coexpression with GGA-WT because we obtained similar results by expression of GLUT4-F5A alone (34.0% ± 6.0% and 89.0% ± 1.0% for basal and insulin, respectively) or together with empty vector (29.0% ± 3.0% and 91.0% ± 3.0% for basal and insulin, respectively) in 3T3L1 adipocytes. Nevertheless, similar to GLUT4-WT, the plasma membrane localization of GLUT4-F5A was significantly inhibited by coexpression of GGA-DN both in the insulin and the basal state, which suggests that this motif is not specifically required for the GGA-dependent regulation of GLUT4 trafficking. As expected, mutation of the di-leucine motif within the carboxyl terminus of GLUT4 to alanine (GLUT4-SAA) had no effect on basal or insulin-stimulated GLUT4 translocation, and this mutant was also fully inhibited by coexpression of GGA-DN in the same degree as GLUT4-WT (Fig. 8A).

The insulin-dependent translocation of the expressed GLUT4-WT reporter was confirmed by subcellular fractionation and immunoblotting (Fig. 8B). Sixteen hours after transfection, GLUT4-WT was most abundant in the low-speed pellet fraction and the high-speed pellet fraction with low levels in the plasma membrane fraction in the basal state. However, insulin stimulation resulted in decreased GLUT4-WT levels in the high-speed pellet fraction concomitant with an increase in the plasma membrane (*top left panel*). Similar results were obtained in adipocytes cotransfected with GGA-WT and GLUT4-WT (*middle left panel*). Consistent with the confocal fluorescence data, coexpression of GLUT4-WT with GGA-DN markedly reduced the GLUT4 levels in the high-speed pellet fraction and prevented the insulin-stimulated translocation to the plasma membrane fraction (*bottom left panel*). In contrast, the GLUT4-F5A mutant had a similar intracellular distribution as GLUT4-WT in the basal state when either alone or cotransfected with GGA-WT but displayed a higher basal level in the plasma membrane fraction (*top* and *middle right panels*). Although insulin was still able to induce a moderate degree of GLUT4-

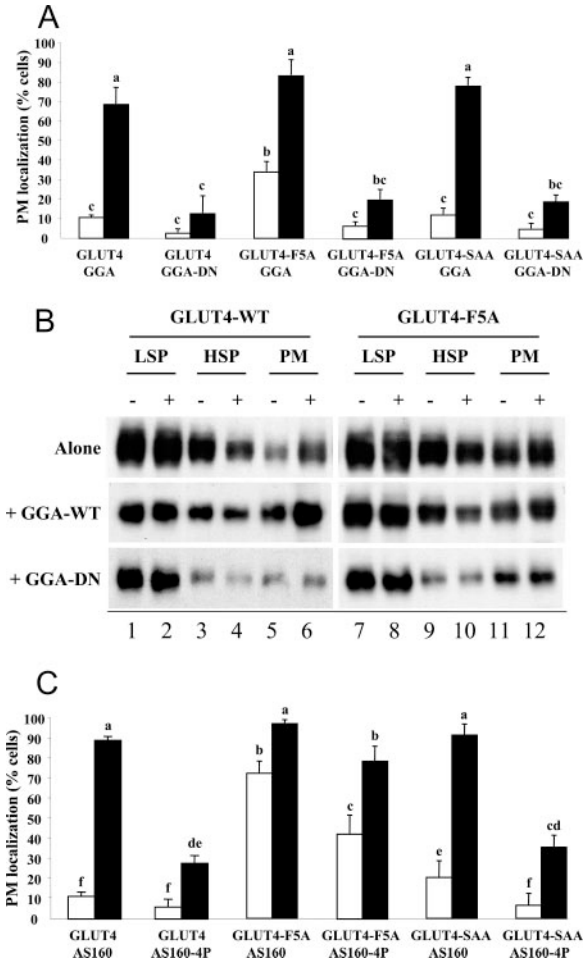

Fig. 8. GGA and AS160 Sensitivity of the GLUT4-F5A and GLUT4-SAA Point Mutants

A, Differentiated 3T3L1 adipocytes were cotransfected with 50 μg each of a GLUT4-EGFP construct and DsRed-GGA-WT or DsRed-GGA-DN. After an overnight incubation period, the cells were either insulin stimulated (100 nM for 30 min) or left untreated as described in *Materials and Methods*. *White bars*, basal; *black bars*, insulin. Values are presented as percentage (mean ± SEM) of cells showing a plasma membrane (PM) ring obtained by counting 100 GGA-positive cells per condition and averaged from three independent experiments. B, Differentiated 3T3L1 adipocytes were co-electroporated with 50 μg each of myc-GLUT4-EGFP or myc-GLUT4-F5A-EGFP without (*upper panel*) or with GGA-WT (*middle panel*) or GGA-DN (*lower panel*) and allowed to recover for 16 h. Transfected cells were serum starved for 2 h followed by treatment without (lanes 1, 3, 5, 7, 9, and 11) or with (lanes 2, 4, 6, 8, 10, and 12) 100 nM insulin for 30 min. Cells were then collected, lysed, and subjected to differential centrifugation as described previously (32). The membrane fractions collected above were subjected to Western blotting for the detection of GLUT4-WT and GLUT4-F5A as described in *Materials and Methods*. HSP, High-speed pellet; LSP, low-speed pellet; PM, plasma membrane. C, Differentiated 3T3L1 adipocytes were cotransfected with 50 μg each of the GLUT4-EGFP constructs and Flag-AS160-WT or Flag-AS160-4P, and after an overnight incubation, period the cells were either insulin stimulated (100 nM for 30 min) or left untreated as described in A above. In A and C, statistical analysis was performed using a one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. *Identical letters* indicate *bars* that are not statistically different from each other ($P < 0.05$).

F5A translocation to the plasma membrane fraction, this was markedly reduced compared with GLUT4-WT. In addition, coexpression of GLUT4-F5A with GGA-DN markedly reduced the levels of GLUT4-F5A in the high-speed pellet and plasma membrane fractions compared with cells with GLUT4-F5A alone or cotransfected with GGA-WT (*bottom right panel*). Based upon subcellular fractionation, insulin again had no significant effect on GLUT4-F5A translocation in the presence of GGA-DN, similar to GLUT4-WT with GGA-DN cotransfected cells. Together these data support a model in which GLUT4-F5A trafficking out of the Golgi/*trans*-Golgi network to the IRC is dependent on GGA function.

We next examined the effect of AS160 on GLUT4-F5A and GLUT4-SAA point mutations translocation because AS160 has been reported to play an important functional role in the regulation of GLUT4 exit from the IRC (19, 23, 25). As shown in Fig. 8C, the dominant-interfering AS160-4P significantly inhibited the insulin-stimulated translocation of GLUT4-WT to the plasma membrane. Furthermore, GLUT4-F5A also showed reduced plasma membrane localization when coexpressed with AS160-4P in comparison with AS160-WT; however, these levels of translocation were still significantly higher when compared with GLUT4-WT, suggesting that GLUT4-F5A was at least partially refractory to this inhibition by AS160-4P (Fig. 8C). Again as expected, both the basal and insulin-stimulated GLUT4-SAA translocation was inhibited by AS160-4P in a manner similar to GLUT4-WT (Fig. 8C). These data demonstrate that the FQQI motif is not involved in the GGA-dependent sorting of the newly synthesized GLUT4 into the IRC, but it is required for the AS160-dependent retention because the GLUT4-F5A mutant is no longer sequestered in the IRC and defaults to the cell surface, bypassing the AS160 regulatory step.

The GLUT4 FQQI Motif Is the Primary Domain Required for Endocytosis and Intracellular Retention

Previous studies have reported that either the FQQI or the SLL motifs are necessary for GLUT4 endocytosis (12, 13, 16, 35). Although our chimera data indicated that the carboxyl-terminal domain is not necessary for GLUT4 internalization, it does appear to contribute to the rate of initial endocytosis. We therefore determined the endocytosis profile of the GLUT4-F5A and the GLUT4-SAA point mutants (Fig. 9). The initial rate of endocytosis was reduced for both the GLUT4-F5A and GLUT4-SAA mutants compared with that of GLUT4-WT. Interestingly, this initial reduction was

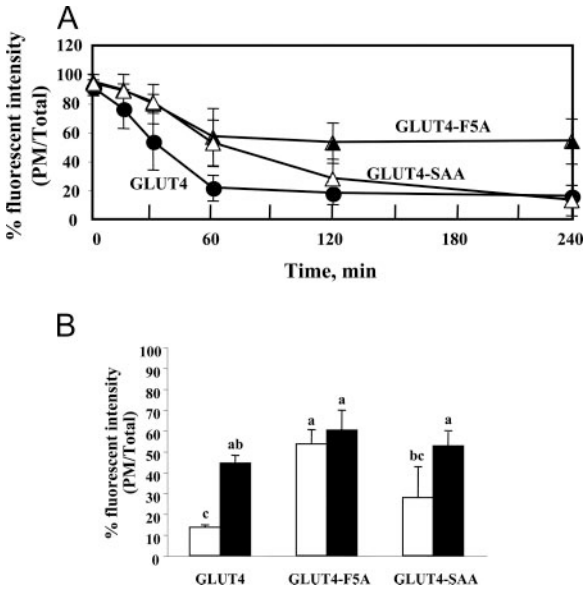

Fig. 9. Determination of Plasma Membrane Endocytosis for the GLUT4-F5A and GLUT4-SAA Point Mutants

A, Differentiated 3T3L1 adipocytes were transfected with 50 μg each of a myc-tagged GLUT4 protein GLUT4-WT (●), GLUT4-F5A mutant (▲), and GLUT4-SAA mutant (△). After an overnight incubation, all the cells were insulin stimulated (100 nM for 30 min) to induce the translocation of all the constructs that were intracellularly sequestered. The cells were then cooled to 4 C, and the exofacial myc tag was labeled with the c-Myc antibody for 1 h. The cells were extensively washed to remove the unbound Myc antibody and residual insulin and then warmed to 37 C to initiate endocytosis. The cells were fixed at 0, 15, 30, 60, 120, and 240 min after warming to 37 C and labeled with a Texas Red fluorescent anti-Myc secondary antibody. B, At the 240-min time point, one set of the GLUT4-WT-, GLUT4-F5A-, and GLUT4-SAA-expressing cells was stimulated with insulin (100 nM for 30 min). The amount of plasma membrane (PM)-localized myc epitope *vs.* total cell myc epitope was quantified as described in *Materials and Methods*. Values are presented as percentage (mean ± SEM) showing the ratio of Texas Red signal intensity in plasma membrane compared with the entire cell fluorescence. Values are presented as percentage (mean ± SEM) from the quantification of 10 cells per time point averaged from two to five independent experiments. Statistical analysis was performed using a one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. *Identical letters* indicate *bars* that are not statistically different from each other ($P < 0.05$).

identical between the two mutants. The difference was that the GLUT4-SAA mutant continued to internalize and reached the same steady state as GLUT4-WT by 4 h as did chimera 1-441. In contrast, the GLUT4-F5A mutant reached a different steady-state equilibrium with only approximately 40% apparently internalized (Fig. 9A). Moreover, after endocytosis and recycling, both the GLUT4-WT and the GLUT4-SAA mutant were capable of responding to a second round of insulin stimulation (Fig. 9B). In contrast, the small amount of internalized and recycled GLUT4-F5A mutant failed to display any significant insulin-stimulated translocation back to the cell surface (Fig. 9B). These data indicate that both the FQQI and SLL motifs participate in the initial rate of GLUT4 endocytosis from the plasma membrane. However, once internalized, the SLL motif is not responsible for intracellular sorting, and instead, other motifs (FQQI) direct GLUT4 to the IRC. Because the FQQI motif is required for AS160 retention, once this mutant is internalized, it fails to undergo intracellular sequestration and defaults back to the plasma membrane.

DISCUSSION

We have previously reported that after initial biosynthesis, the GLUT4 protein enters into the IRC by a relatively slow (6–9 h) Golgi/post-Golgi sorting process without transiting through the plasma membrane (27). This biosynthetic sorting depends upon the coat adaptor protein GGA, which is involved in the intracellular trafficking of several cargo molecules from the *trans*-Golgi network to the endosome system. GLUT4 itself, however, does not directly associate or contain a GGA-binding consensus site, which indicates that an accessory intermediate protein is required. Recently, it has been reported that sortilin may serve in this role (29). We have observed that only the biosynthetic sorting step and not recycling is GGA dependent (32). In any case, it is critical to understand the structural motifs in GLUT4 that are responsible for coupling GLUT4 to the GGA-dependent sorting process into the IRC and those responsible for its exit from the IRC to the plasma membrane. In addition, a key element of the GLUT4 trafficking itinerary is that under both basal and stimulated conditions, GLUT4 is not static but continuously moves through and equilibrates among numerous compartments. Thus, many of the discrepancies in the literature with regard to the localization and functional motifs are likely due to the different experimental paradigms used in these studies. However, in many expression studies the time after expression and the contribution of biosynthetic sorting was not considered, and these particular parameters will have profound effects on the apparent localization of GLUT4.

Nevertheless, based upon these studies, it has been concluded that four motifs account for distinct separate but overlapping trafficking steps (for review see Ref. 8). The FQQI and SLL motifs were predicted to be required for plasma membrane endocytosis and the FQQI motif for further sorting into a pre-GLUT4 storage compartment. Exit from the pre-GLUT4 storage compartment to the degradative pathway via late endosomes was reported to require the carboxyl-terminal PDEND motif, whereas exit to the IRC required the carboxyl-terminal Y502 residue (11). On the other hand the carboxyl-terminal SLL motif functioned in the biosynthetic sorting from the *trans*-Golgi network to the pre-GLUT4 storage compartment (11, 15).

To refine this model and to functionally identify trafficking motifs that interact with known or still unidentified trafficking effectors necessary for insulin responsiveness in adipocytes, we have performed a detailed analysis of the biosynthetic and recycling pathway of GLUT4 into and out of the IRC as schematically represented in Fig. 10. To accomplish this objective, we took advantage of the established functions of two critical regulators of GLUT4 trafficking, GGA that is required for the biosynthetic transport of GLUT4 into the IRC and AS160 that is required for the transport of GLUT4 out of the IRC *en route* to the plasma membrane. Combining these effectors with the expression of various GLUT4/GLUT1 chimeras and GLUT4 point mutations we have been able to establish several motifs that function to sort GLUT4 through specific trafficking steps.

Our data clearly demonstrate that the cytoplasmic amino-terminal region in conjunction with the large intracellular loop between transmembrane domains 6 and 7 is sufficient to recapitulate the full trafficking itinerary of GLUT4 when substituted into the constitutively trafficking GLUT1 isoform. This includes the appropriate GGA-dependent biosynthetic sorting into the IRC, insulin stimulation, AS160-dependent translocation from the IRC, plasma membrane internalization, and recycling back to the IRC. However, both domains are necessary, because none of the GLUT4 single-domain chimeras behaved completely like GLUT4-WT in any of the specific experiments performed. This is because appropriate sorting and retention of GLUT4 in the IRC requires both the amino terminus and large cytoplasmic loop working in concert. Thus, the loss of either one of these domains resulted in a bypass of the GGA and AS160 regulatory steps and default to the cell surface. Several previous studies have reported that the carboxyl-terminal SLL motif plays an important role in GLUT4 endocytosis and have pointed to the amino-terminal FQQI motif without any significant contribution (35–38). We can now account for these apparent differences in interpretation because the initial rate of endocytosis for both the GLUT4/GLUT1 chimera 1-441 and the GLUT4-SAA mutant was reduced compared with GLUT4-WT. Nevertheless, at longer times, both constructs reached the same steady-state intracellular distribution as GLUT4 and were also fully responsive to insulin stimulation. Thus, these data demonstrate that the SLL motif does contribute to the initial rate of plasma membrane endocytosis, but it is not involved in subsequent intracellular sorting and retention. However, because GLUT4 endocytosis still occurs despite the loss of the SLL motif, albeit somehow slower, it indicates that other sequences within the GLUT4 protein provide the more critical trafficking and sorting functions.

In this regard, other studies have suggested that the amino terminus and specifically the FQQI motif plays a critical role in plasma membrane endocytosis (13, 15, 16, 39). Our data are consistent with this domain also contributing to GLUT4 endocytosis; however, the magnitude of its contribution to the initial rate of endocytosis appears to be similar to that of the SLL motif. Importantly, unlike the SLL motif, the FQQI motif is required for intracellular sorting and retention after endocytosis. Furthermore, although this domain is not

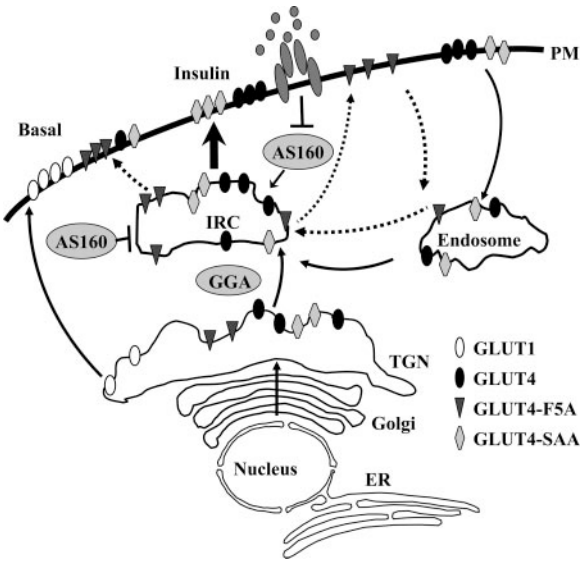

Fig. 10. Schematic Model Illustrating the Intracellular Trafficking of GLUT4

Newly synthesized GLUT4 as well as the GLUT4-F5A and GLUT4-SAA mutants traffic through the Golgi and the *trans*-Golgi network (TGN) and into the IRC in a sorting process that is dependent upon the adaptor protein GGA. In contrast, after biosynthesis, GLUT1 directly defaults from the TGN to the plasma membrane (PM) in a GGA-independent process. In the basal state, GLUT4 is mainly sequestered into the IRC and translocates to the plasma membrane after insulin stimulation. Both steps, sequestration and exit from the IRC, are regulated by the Akt substrate AS160. AS160 functions as a Rab GAP inhibiting a putative Rab protein necessary for GLUT4 exit from the IRC (*left side*). It has been suggested that after insulin-stimulated Akt phosphorylation, the AS160 function is inhibited, thereby allowing the exit of GLUT4 from its intracellular sequestration to the plasma membrane (*right side*). These steps require the FQQI motif within the amino-terminal domain of GLUT4 but not the SLL motif in the carboxyl terminal domain, because in basal conditions, the GLUT4-SAA mutant remains sequestered in the IRC, but the GLUT4-F5A mutant leaks to the plasma membrane. Furthermore, in steady-state conditions, GLUT4 continuously cycles between the plasma membrane and several intracellular compartments. Although the FQQI and the SLL motifs are implicated in GLUT4 endocytosis, the FQQI motif, not the SLL motif, is also required for GLUT4 recycling back through the endosome system to the IRC. In addition, this schematic model also shows that the point mutants GLUT4-F5A and GLUT4-SAA undergo different rates of endocytosis and enter different recycling pathways; that is, GLUT4-F5A recycles back to the cell surface after endocytosis due to loss of AS160 sensitivity, whereas, like GLUT4-WT, GLUT4-SAA is able to go back and be sequestered in the IRC, because the SLL motif is not required for sorting GLUT4 back to the IRC. ER, Endoplasmic reticulum.

necessary for GGA sensitivity, it is required for AS160 sensitivity because the GLUT4-F5A mutant displayed a lack of retention in the IRC. Thus, the high basal state accumulation of the GLUT4-F5A mutant results from a combination of a reduced rate of endocytosis coupled with a lack of appropriate intracellular retention due to the loss of AS160 sensitivity. It remains to be determined whether this results from an inability of GLUT4-F5A to interact with AS160/AS160 effectors or that this mutant completely bypasses the AS160 functional IRC compartment and defaults to the general endosome trafficking pathway.

On the other hand, our data also demonstrate that the large cytoplasmic loop plays an important functional role in concert with the amino-terminal domain to fully recapitulate all the appropriate trafficking properties of GLUT4, at least when placed in the context of the constitutively trafficking GLUT1 protein. However, further deletion analysis of this domain was inconclusive, indicating that this region must undergo appropriate folding and that the three-dimensional structure is a critical factor in mediating the interaction with the amino-terminal domain and/or independent regulation of the sorting process. More studies will now be required to identify the functional interacting partners to both the amino terminus and large cytoplasmic loop of GLUT4 responsible for the GGA-dependent sorting to the IRC and AS160-dependent exit to the plasma membrane.

MATERIALS AND METHODS

Plasmids

The GLUT4-WT and GLUT1-WT cDNAs as well as the GLUT4 point mutations and GLUT4/GLUT1 chimera constructs were generated as EGFP fusion proteins by overlapping PCR procedures as described previously (31). In addition, three myc tags were added to some constructs in the extracellular loop between transmembrane domains 1 and 2 to use in the endocytosis experiments. All final constructs were sequenced in their entirety to check for unexpected mutations. A schematic representation of the different constructs generated is shown in Fig. 1. The GLUT4/GLUT1 chimeras consist of exchanges between the regions corresponding to the three main intracellular domains: amino terminus, intracellular loop, and carboxy terminus as previously described (31). The nomenclature used for these chimeras is based on the form x-xxx, with the first number (1 or 4) representing the parental protein backbone, GLUT1 or GLUT4, whereas the numbers on the right indicate to which isoform, GLUT1 or GLUT4, corresponds each one of the three intracellular regions exchanged. The point mutations F5A and SAA consist of the mutation to alanine (A) of amino acids F^{5} and $L^{489}L^{490}$, respectively. The constructs of DsRed-GGA1 wild type (GGA-WT) and DsRed-GGA dominant-interfering mutant (GGA-DN) consisting on the VHS-GAT domains of GGA1 were generated as previously described (32). HA-GGA-DN was generated by subcloning the VHS-GAT domains of GGA1 into a pKH3 vector. The Flag-tagged AS160 wild type (AS160-WT) and dominant-interfering mutant (AS160-4P) cDNAs were a kind gift of Dr. Gustav Lienhard (Dartmouth University).

Culture and Transient Transfection of 3T3L1 Adipocytes

Murine 3T3L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured at 37 C and 8% CO_2 and differentiated into adipocytes. Fully differentiated 3T3L1 adipocytes were transiently transfected by electroporation under low-voltage conditions (160 V, 950 μF) with 50 μg each of the different DNA plasmids as described previously (31).

Analysis of GGA and AS160 Sensitivity

After an overnight incubation period (18 h), differentiated 3T3L1 adipocytes expressing the different GLUT-EGFP-tagged plasmids plus the wild-type or dominant-interfering DsRed-GGA or Flag-tagged AS160 construct were serum starved in DMEM for 2–2.5 h before each experiment. Cells were then incubated with or without insulin (100 nM) for 30 min, fixed, and finally mounted in Vectashield medium (Vector Laboratories, Burlingame, CA). AS160 expression was detected by immunofluorescence using Flag M2 monoclonal antibody (1:500) and secondary antimouse Texas Red-conjugated antibody in permeabilizing conditions. The translocation of the different GLUT4 constructs to the plasma membrane was analyzed by wide-field fluorescent microscopy and presented (mean ± SEM) as previously described (31). For the Western blot analysis, protein lysates in RIPA buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, pH 7.4) supplemented with a 1:1000 dilution of a protease inhibitors cocktail (P8340; Sigma Chemical Co., St. Louis, MO) were obtained from 3T3L1 adipocytes cotransfected with the different GLUT4-EGFP plus the DsRed-GGA or Flag-tagged AS160 constructs after an overnight (18 h) incubation period. Thirty micrograms of total protein (measured with the BCA protein assay kit from Pierce, Rockford, IL) were separated by 7.5% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane. Western blot analysis was performed using the following primary antibodies: rabbit polyclonal anti-GFP (Clontech, Mountain View, CA) for GLUT4 constructs, mouse monoclonal M2 anti-Flag (Sigma) for AS160, mouse monoclonal anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA) for GGA-DN, and custom-made rabbit polyclonal anti-rat GGA1 (amino acids 289–508) for GGA-WT from Covance (Princeton, NJ) and anti-rat GLUT4 (AlO2, amino acids 467–509) for endogenous GLUT4 from Covance.

Subcellular Fractionation

Adipocytes were coelectroporated with 50 μg each of myc-GLUT4-EGFP or myc-GLUT4-F5A-EGFP with or without GGA-WT or GGA-DN and allowed to recover for 16–18 h before serum starvation (2–2.5 h) and insulin stimulation (100 nM, 30 min). Cells were then washed in PBS, resuspended in HEPES-EDTA-sucrose buffer and sheared by 10 passes through a ball-bearing homogenizer. Differential centrifugation was used to fractionate adipocytes as described previously (32, 40, 41). Total protein in the high-speed pellet, low-speed pellet, and plasma membrane fractions was quantified using the BCA protein assay kit (Pierce). Equal protein amounts (10 μg) from each of the subcellular fractions were loaded onto 7.5% SDS-polyacrylamide gels, subjected to electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with a polyclonal GFP antibody to detect GLUT4 constructs.

GLUT4 Endocytosis

Adipocytes that were transfected with the different myc-tagged GLUT-EGFP constructs were incubated in serum-free medium for 3 h. The cells were then treated with 100 nM

insulin for 30 min to translocate the reporters to the cell surface followed by cooling the cells at 4 C to block subsequent trafficking. The cell surface-exposed reporters were then labeled with a c-Myc antibody (Santa Cruz; 9E10, 1:50) for 1 h, and cells were washed to remove insulin and excess c-Myc antibody and returned to 37 C for various times to initiate endocytosis. Then the cells were fixed in 4% paraformaldehyde at room temperature for 10 min, blocked with 5% donkey serum, and incubated with Texas Red-conjugated antimouse secondary antibody (1:500) for 45 min at 37 C under permeabilizing conditions. The slides were then mounted in Vectashield medium and examined by confocal-laser scanning microscopy. Endocytosis was quantified by dividing the plasma membrane surface Texas Red fluorescent intensity by the total cell content of Texas Red fluorescent intensity in 10 cells for each time point per experiment. The mean value was calculated from the average of three independent experiments.

The ability of GLUT4 to undergo a second round of insulin-stimulated translocation after endocytosis was determined by labeling the exofacial myc-GLUT4-EGFP with the myc antibody at 4 C and allowing the cells to undergo endocytosis for 4 h at 37 C. The cells were then treated in the absence and presence of 100 nM insulin for 30 min, fixed, and labeled with the secondary antimouse antibody with permeabilization.

Statistical Analyses

Translocation data are presented as mean ± SEM. The data from Figs. 4–6, 8, A and C, and 9B were analyzed by one-way ANOVA followed by the Student-Newman-Keuls multiple-range test. Statistical analyses were made at a significance level of $P < 0.05$ using the SPSS program (version 13.0; SPSS, Chicago, IL).

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