Analysis of Amino and Carboxy Terminal GLUT-4 Targeting Motifs in 3T3-L1 Adipocytes Using an Endosomal Ablation Technique†

Derek R. Melvin,‡ Brad J. Marsh,§,|| Adrian R. Walmsley,⊥ David E. James,§ and Gwyn W. Gould*,‡

Divisions of Biochemistry and Molecular Biology and of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, and Centre for Molecular and Cellular Biology and Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland 4068, Australia

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ABSTRACT: The targeting of the insulin-responsive glucose transporter, GLUT-4, to an intracellular compartment in adipocytes and muscle is one of the key features responsible for the unique insulin sensitivity of this transporter. Through expression of epitope-tagged GLUT-4 mutants in 3T3-L1 adipocytes, two motifs have been identified as playing a central role in GLUT-4 targeting: FQQI in the amino terminus and a di-leucine motif in the carboxy terminus. The goal of this study was to explore the role of these targeting motifs in the intracellular sorting of GLUT-4 using the Tf−HRP ablation technique. This technique provides a quantitative assessment of the amount of GLUT-4 located in recycling endosomes. In basal adipocytes, we find that ∼40% of GLUT-4 is ablated following Tf−HRP loading. In contrast, here we demonstrate that the intracellular pool of a mutant in which F^5 was mutated to A^5 is localized to the recycling endosomal pathway, suggesting that the amino terminal FQQI motif functions in trafficking GLUT-4 from early endosomes. In contrast, GLUT-4 in which $L^{489}L^{490}$ was mutated to $A^{489}A^{490}$ was localized predominantly to a nonablated compartment. These data imply a role for the di-leucine motif in sorting from a separate intracellular compartment, such as the TGN. Our findings are discussed within the context of a revised multicompartment model for GLUT-4 trafficking in adipocytes, in which mutations in either the FQQI or LL motifs result in the altered subcellular trafficking of GLUT-4 between multiple intracellular compartments.

Insulin stimulates glucose uptake into adipocytes and muscle by virtue of the specific expression of an insulin-regulatable glucose transporter, GLUT-4[1] (*1*−*5*). The translocation of GLUT-4 from an intracellular site to the plasma membrane in insulin-exposed cells is largely responsible for the 20−30-fold increase in the extent of glucose transport observed in these cells (*4*−*9*). Under basal conditions, >95% of GLUT-4 is localized to tubulovesicular elements that are found clustered adjacent to early or late endosomes, in the trans-Golgi reticulum (TGN), or in the cytoplasm often close to the plasma membrane (*4*, *5*). The level of GLUT-4 is reduced by 40−50% at each of these locations following insulin treatment, suggesting that all of these compartments participate in the insulin-regulated recycling of GLUT-4 (*4*, *5*).

Ectopic expression of GLUT-4 in a variety of cell types has shown that the primary sequence of this protein contains information that directs its intracellular sequestration (*10*−*12*). This is in contrast to other members of the glucose transporter family, which are expressed primarily at the cell surface (*11*, *13*−*16*). Several laboratories have attempted to define the molecular features of GLUT-4 which dictate its intracellular sequestration and unique targeting. These studies have identified two motifs, both of which are found within the cytoplasmic domains of GLUT-4, and which appear to regulate the unique intracellular distribution of this protein. (*14*, *17*−*22*).

The first of these motifs (F^5QQI^8) is found within the amino terminal cytoplasmic domain of the protein (*17*, *18*, *22*). Interestingly, this motif resembles tyrosine-based internalization motifs identified in plasma membrane proteins which undergo endocytosis, such as the mannose 6-phosphate receptors (MPRs) and the transferrin receptor (TfR) (*23*−*25*). Present knowledge suggests that there may be further subclasses of aromatic-based endocytic signals (*26*), and it

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* To whom all correspondence should be addressed. Telephone: 44-141-330-5263. Fax: 44-141-330-4620. E-mail: gbca71@udcf.gla.ac.uk.

‡ Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow.

§ University of Queensland.

|| Current address: Boulder Laboratory for Three-Dimensional Fine Structure, Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO 80309-0347.

⊥ Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow.

[1] Abbreviations: GLUT, glucose transporter isoform; DME medium, Dulbecco's modified Eagle's medium; TGN, trans-Golgi reticulum; PM, plasma membrane; LDM, low-density microsomes; HDM, high-density microsomes; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; TfR, transferrin receptor; CHO, Chinese hamster ovary; Tf−HRP, transferrin−HRP conjugate; DAB, 3,3′-diaminobenzidine; VAMP-2, vesicle-associated membrane protein-2 (synaptobrevin 2).

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is possible that the phenylalanine-based motif identified within GLUT-4 may be a further example of such signals. The second region identified within GLUT-4 as being important in regulating its subcellular distribution is contained within its carboxy terminal cytoplasmic domain, and comprises a di-leucine motif (*19*, *20*, *27*). Di-leucine (or isoleucine-leucine) motifs have been shown to function as endocytic signals in other proteins (*28*−*30*). In the context of GLUT-4, it has been suggested that both of these motifs regulate internalization of the protein from the plasma membrane (*21*, *22*, *31*). However, it is not known if they function in other aspects of GLUT-4 trafficking. Previous studies have shown that when expressed in adipocytes, GLUT-4 in which either F^5 or L^{489} and L^{490} were mutated to alanines is targeted to the cell surface aberrantly (*22*). This could be due to a reduced level of either internalization or intracellular sequestration.

We have previously described a technique ("compartment ablation") which enables greater resolution of the intracellular compartments containing GLUT-4 in adipocytes (*32*, *33*). Using this technique, we have shown that in resting 3T3-L1 adipocytes, GLUT-4 is segregated into at least two discrete intracellular pools. The first corresponds to recycling endosomes and contains ~40% of the intracellular GLUT-4. The second compartment, which is devoid of recycling transferrin receptors, contains ~60% of the intracellular GLUT-4 and is not ablated after Tf−HRP loading.

Thus, in this study, we set out to determine if disruption of either the FQQI motif in the GLUT-4 amino terminus or the di-leucine motif in the carboxy terminus altered the distribution of GLUT-4 between the recycling and the nonablated compartments. Our results show that mutation of L^{489} and L^{490} to alanines does not prevent GLUT-4 from reaching the nonablated compartment, but greatly reduces the amount of GLUT-4 present in the recycling endosomal compartment. In contrast, the FQQI mutant is predominantly located in the recycling compartment and fails to accumulate in the nonablated compartment under steady-state conditions.

MATERIALS AND METHODS

Materials. All reagents were described previously (*22*, *33*, *34*).

Cell Culture. 3T3-L1 fibroblasts were grown and differentiated as described in ref *35* and used 8−12 days postdifferentiation. Before use, cell monolayers were washed with serum-free DME and then incubated in serum-free DME for 2 h. The methodology describing in detail the construction of human GLUT-3 epitope-tagged transporter cDNAs, and their stable transfection into 3T3-L1 fibroblasts, has been described elsewhere (*22*).

Expression Levels of Recombinant GLUT-4 Constructs in Adipocyte Cell Lines. The constructs employed in this study have been characterized in 3T3-L1 fibroblasts and adipocytes previously by subcellular fractionation and indirect immunofluorescence microscopy (*22*). Briefly, multiple clonal cell lines expressing recombinant GLUT-4 constructs at a range of expression levels were classified into two broad categories: low expressors, in which total GLUT-4 expression was at a level comparable to that of endogenous GLUT-4 in untransfected adipocytes, and high expressors, where the total expression level was >4-fold higher than that of endogenous GLUT-4 expressed by nontransfected adipocytes. Hence, we have selected specific cell lines for more detailed analysis on the basis of the following points.

(1) The distribution of epitope-tagged wild-type GLUT-4 (TAG) is indistinguishable from that of endogenous GLUT-4 in different cell lines with markedly different expression levels (*22*). We have examined the clone expressing the highest levels of TAG (TAG 3B1), as the intracellular sequestration of TAG in this cell line is maintained despite a level of total GLUT-4 expression approximately 6-fold greater than that observed in wild-type cells. Thus, we aimed to determine whether any evidence for the saturation of intracellular trafficking pathways could be found at this level of overexpression using the techniques of endosomal ablation.

(2) The aberrant targeting of GLUT-4 containing the F^5 to A^5 mutation (FAG) is independent of expression level (*22*). Thus, we have chosen two clones expressing FAG at low levels, i.e., comparable to the level of endogenous GLUT-4 in nontransfected adipocytes (FAG 3C2 and FAG 2C5), to examine the effects of this mutation upon the intracellular distribution of GLUT-4.

(3) The targeting of GLUT-4 containing the $L^{489}L^{490}$ to $A^{489}A^{490}$ mutation (LAG) in adipocytes is dependent upon the level at which it is expressed (*22*). Thus, two clones with markedly different expression levels (LAG 1A5 and LAG 1D3, high and low expressors, respectively) were selected for further analysis by endosomal ablation. Figure 1 presents a schematic illustration of these mutants, and their relative expression levels are listed in Table 1. TAG was distributed among subcellular fractions of 3T3-L1 adipocytes in a fashion identical to that of endogenous GLUT-4 (*22*). In contrast, FAG was predominantly expressed at the plasma membrane, independently of expression level. At low to moderate expression levels, LAG was indistinguishable from endogenous GLUT-4, but at higher levels of expression was found to accumulate at the plasma membrane. For full details, see ref *22*.

Preparation and Use of HRP-Conjugated Transferrin. The transferrin−horseradish peroxidase conjugate (Tf−HRP) was prepared, purified, and iron-loaded exactly as described previously (*33*). Following preincubation for 2 h in serum-free DME, adipocytes were incubated with 20 μg/mL Tf−HRP for the times indicated to fill the TfR itinerary. Cells were then washed in ice-cold isotonic citrate buffer [150 mM NaCl and 20 mM sodium citrate (pH 5.0)] and kept on ice to prevent further vesicle trafficking during the DAB cytochemistry reactions (see below). Cell surface-attached Tf−HRP was removed by acid washing three times over the course of 10 min in ice-cold citrate buffer. Cells were then washed in ice-cold PBS. DAB was added at a concentration of 100 μg/mL to all cells, and H_2O_2 was added (0.02%) to one of each pair of wells. After incubation for 60 min at 4 °C in the dark, the reaction was stopped by washing in PBS containing 5 mg/mL BSA. This was then aspirated, and the samples were prepared for immunoblotting. For all experiments, duplicate plates were employed, one of which was exposed to DAB and H_2O_2, and the other to only DAB as a negative control (*32*, *33*, *36*).

Subcellular Fractionation of Adipocytes. Adipocytes were subjected to differential centrifugation as described previously (*33*, *37*). Cells grown on 10 cm cell culture dishes

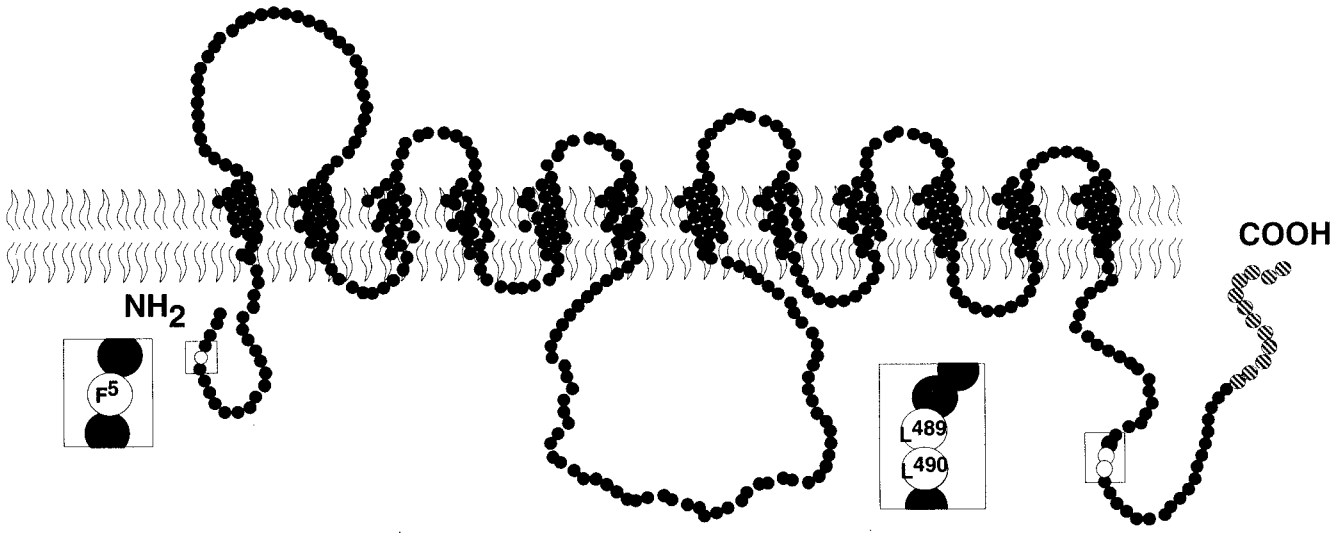

FIGURE 1: Schematic representation of the recombinant GLUT-4 constructs employed in this study. To discriminate between recombinant and endogenous GLUT-4 in stably transfected 3T3-L1 adipocytes, a foreign epitope encompassing the 12 carboxy terminal amino acid residues from human GLUT-3 was introduced at the extreme carboxy terminus of GLUT-4. Wild-type GLUT-4 epitope-tagged in this fashion is referred to as TAG. Epitope-tagged GLUT-4 containing point mutations at either F^5 or L^{489} and L^{490} is referred to as FAG or LAG, respectively. Amino acid residues are represented using the single-letter code.

Table 1: Effect of Ablation on Intracellular GLUT-4 Levels[a]

species	expression level	% signal remaining after ablation	
		1 h at 37 °C	3 h at 37 °C
wild-type GLUT-4	1.96	64 ± 3	59 ± 8
TAG 3B1	11.48	62 ± 8	60 ± 9
FAG 3C2	3.88	56 ± 7	25 ± 10
FAG 2C5	2.64	53 ± 5	18 ± 9
LAG 1A5	8.25	96 ± 7	98 ± 3
LAG 1D3	2.54	92 ± 8	89 ± 7

[a] The relative levels of total GLUT-4 expression (arbitrary units) in the cell lines examined in this study have been determined previously (*22*) and are presented above for comparative purposes. Clones were classified into two broad categories on the basis of the total level of GLUT-4 expression relative to the level of endogenous GLUT-4 expressed by wild-type 3T3-L1 adipocytes. TAG 3B1 and LAG 1A5 are high-expressor clones. FAG 3C2, FAG 2C5, and LAG 1D3 were classified as low expressors. Duplicate sets of 10 cm plates of adipocytes were loaded with Tf−HRP for 1 or 3 h at 37 °C. After this time, the DAB cytochemistry was performed as described, with hydrogen peroxide added to one plate but not the other. LDM membranes were prepared, and the GLUT-3 immunoreactive signal was quantified. The difference in signals between the plates incubated with or without peroxide represents the extent of protein ablation (see Figure 3). Shown above is the signal remaining in the LDM *after* ablation expressed as a percentage of the signal in the LDM *before* ablation (mean ± SD determined from three independent experiments). In all experiments, an additional control experiment was performed in which cells were incubated with Tf−HRP at 4 °C and then ablated. Under these conditions, no internalization of Tf−HRP is expected, and consistent with this, we observed no ablation of either the recombinant GLUT-4 constructs (see Figure 3) or endogenous GLUT-4 (*33*). Values for endogenous GLUT-4 were from plates of nontransfected adipocytes, measured using the same batches of conjugate employed for the mutants.

were scraped into ice-cold HES buffer [20 mM Hepes, 1 mM EDTA, and 255 mM sucrose (pH 7.4); 5 mL per 10 cm plate] containing protease inhibitors (1 μg/mL pepstatin A, 0.2 mM diisopropyl fluorophosphate, 20 μM E-64, and 50 μM aprotinin) and were homogenized by 10 strokes of a Teflon/glass homogenizer. The homogenate was sedimented at 19000*g* for 20 min at 4 °C. The pellet from this spin was resuspended in 2 mL of HES, layered onto 1 mL of 1.12 M sucrose in HES, and sedimented at 100000*g* for 60 min at 4 °C in a swing-out rotor. Plasma membranes were collected from the interface by careful aspiration, resuspended in HES, and collected by sedimentation at 41000*g* for 20 min at 4 °C. The supernatant from the 19000*g* spin was recentrifuged at 41000*g* to yield a high-density microsomal (HDM) pellet, and the supernatant from this spin was sedimented at 180000*g* for 75 min at 4 °C to collect low-density microsomes (LDM). All fractions were resuspended in equal volumes of HES buffer (cell equivalents), snap-frozen in liquid nitrogen, and stored at −80 °C.

Sucrose Gradient Centrifugation. LDM membranes were rehomogenized gently in a small volume of HES buffer. Aliquots of this fraction were loaded onto step gradients comprised of 1.5 to 0.5 M sucrose solutions prepared in 20 mM Hepes and 1 mM EDTA (pH 7.4). These were then centrifuged at 75000*g* for 24 h at 4 °C. Samples were collected by tube puncture, and the protein in each fraction was precipitated. Samples were resuspended in SDS−PAGE buffer and stored at −80 °C prior to analysis (*33*).

Electrophoresis and Immunoblotting. Proteins were electrophoresed on 7.5, 10, or 15% SDS−polyacrylamide gels and transblotted onto nitrocellulose or PVDF sheets as described previously (*34*). Immunolabeled proteins were visualized using either ^{125}I-labeled goat anti-rabbit second antibody or a HRP-conjugated secondary antibody/ECL system and quantitated by either γ-counting or densitometry. The data describing the extent of ablation for the mutant GLUT-4 species (Table 1) are presented as the immunoblot signal remaining in the LDM after ablation expressed as a percentage of the signal in the LDM before ablation. Note that in these experiments, a linear response of the antibody under the conditions used was determined by loading 1× and 0.5× protein loads in adjacent lanes of the gels or blots to ensure the linearity of the signal. Different exposure times were employed for FAG compared to LAG or TAG clones because the levels of FAG expression in intracellular membranes are lower than that of either TAG or LAG (*22*).

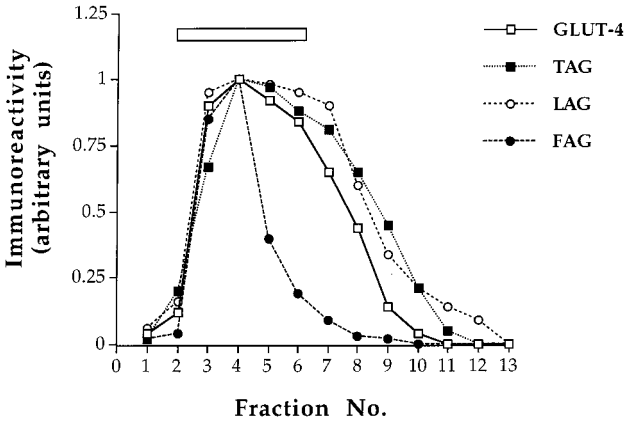

FIGURE 2: Buoyant density analysis of TAG, FAG, and LAG. LDM membranes were prepared and subjected to centrifugation on discontinuous sucrose density gradients. The distributions of TAG, FAG, and LAG were compared to that of endogenous GLUT-4. To directly compare between clones, data were normalized to the fraction containing the greatest level of immunoreactivity. Data are presented as the mean immunoreactivity in each fraction ± SD (arbitrary units) determined from two gradients for each of the two FAG clones, the two LAG clones, and TAG 3B1. The box represents fractions which contain >80% of the early endosomal markers, Rab5 and the TfR (*33*). Fraction 1 has the highest density and fraction 13 the lowest.

Antibodies. The anti-GLUT-4 antibodies used were a rabbit polyclonal antibody raised against a peptide comprising the 14 carboxy terminal amino acid residues of the human isoform of GLUT-4 (*34*) or the corresponding region of the human isoform of GLUT-3 (*38*). The anti-transferrin receptor antibody was from Upstate Biotechnology Inc. (Lake Placid, NY).

RESULTS AND DISCUSSION

The goal of these studies was to determine the partitioning of different GLUT-4 targeting mutants (Figure 1) between discrete intracellular compartments segregated on the basis of their sensitivity to ablation using Tf−HRP. Thus, all of the experiments described here involve the use of the intracellular membrane fraction of adipocytes, referred to as LDM. This fraction is highly enriched in the intracellular GLUT-4 compartment(s) as indicated by the high levels of GLUT-4 and TAG in this fraction (*22*). The LDM fraction also contains reasonably high levels of LAG, whereas the levels of FAG are much lower. This is not a consequence of low expression levels of FAG (see Table 1), but rather, it reflects the very high level of accumulation of FAG in the plasma membrane under basal conditions (*22*). We employed multiple clonal cell lines expressing each mutant at a range of expression levels (see Materials and Methods) which have been previously characterized (*22*).

Initially, to characterize the intracellular distribution of the GLUT-4 mutants with respect to endogenous GLUT-4, we performed sucrose density gradient analysis (Figure 2). When the LDM from basal cells was subjected to this analysis, one single peak of GLUT-4 is resolved in fractions 3−9. Although such an analysis does not provide detailed information about the intracellular compartments which contain the exogenous GLUT-4, the distribution of TAG and LAG among intracellular membranes was indistinguishable from that of endogenous GLUT-4, suggesting that regardless of expression level, these species were targeted to similar membrane compartments. In contrast, FAG exhibited a more restricted distribution (fractions 3−5), even after prolonged exposure of the immunoblots. The fractions containing ∼85% of FAG were also enriched in the early endosomal protein, Rab5 (*33*), indicating that the intracellular distribution of FAG may be restricted compared to that of either endogenous GLUT-4, TAG, or LAG. The shift in distribution of FAG is not a consequence of overexpression, because the clones used expressed FAG at a level similar to that of endogenous GLUT-4. Thus, these results suggest that FAG is preferentially localized to an endosomal compartment in basal adipocytes.

We recently described a procedure in which Tf−HRP is used to selectively "ablate" the recycling compartment of 3T3-L1 adipocytes (*32*, *33*). This approach relies on TfR-mediated delivery of Tf−HRP to the recycling endosomal system. Subsequent exposure of the cells to DAB and hydrogen peroxide generates a cross-linking species within the lumen of compartments which have taken up the Tf−HRP, and results in their subsequent ablation. We have shown that this procedure selectively ablates markers of the recycling endosomal system (TfR, Rab5, and cellubrevin), with little or no effect on TGN or lysosomal markers (TGN38 and lgp120) (*32*, *33*). We find that ∼40% of GLUT-4 is reproducibly ablated. This suggests that GLUT-4 is distributed among at least two compartments, recycling endosomes and what we have referred to as a postendosomal storage compartment (∼60%) (*32*, *33*). Here, we have exploited this technique to examine whether $F^5 \rightarrow A$ or $L^{489}L^{490} \rightarrow AA$ mutations alter the distribution of GLUT-4 between the ablated (endosomal) and nonablated pools.

TAG 3B1 exhibited a pattern of ablation identical to that previously reported for endogenous GLUT-4 (Table 1) (*33*). This provides further evidence that the recombinant epitope-tagged GLUT-4 construct is targeted correctly, and further indicates that GLUT-4 targeting is maintained over a broad expression range, because TAG 3B1, the clone used for these studies, expresses GLUT-4 at a level >6-fold higher than that of endogenous GLUT-4 expression in wild-type cells (Table 1). In contrast, both FAG and LAG mutants exhibited altered patterns of ablation. Two clonal cell lines expressing FAG at low levels, or LAG at low and high levels, were independently examined and gave identical results for each mutant (Table 1). This strongly suggests that the results do not reflect overexpression or clonal variation for either of the constructs. For each clone, we have shown that the ablation conditions employed resulted in quantitative ablation of the TfR (see, for example, Figure 3B).

Using the Tf−HRP ablation procedure, we have observed that the intracellular fraction of FAG is ablated to an extent far greater than either TAG (Figure 3a and Table 1) or endogenous GLUT-4 in wild-type cells (*33*). This suggests that within the cell FAG is confined to a TfR-containing compartment, presumably recycling endosomes, and further suggests that FAG either does not gain access to or does not accumulate in the nonablated compartment.

In contrast to that observed with FAG, the ablation efficiency of LAG expressed at either low or high levels was markedly reduced compared to that of either TAG or wild-type GLUT-4, implying that this mutant is excluded from the recycling endosomal system (Figure 3a and Table

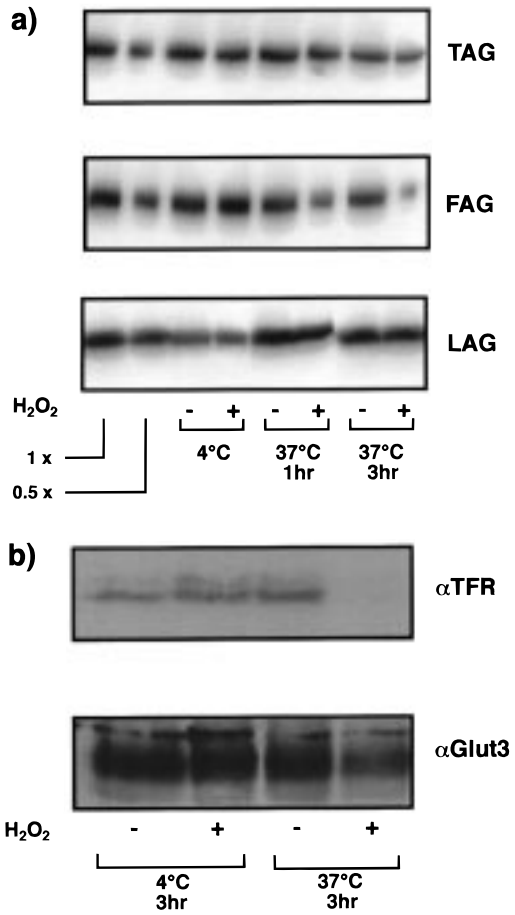

FIGURE 3: Compartment ablation analysis of TAG, FAG, and LAG. LDM membranes were prepared from 3T3-L1 adipocytes loaded with Tf−HRP for 1 h at 4 °C, 1 h at 37 °C, or 3 h at 37 °C, before and after ablation (without and with hydrogen peroxide) as indicated. In panel a, representative immunoblots are shown for representative clones of TAG, FAG, and LAG (TAG 3B1, FAG 3C2, and LAG 1A5). In these experiments, cells were loaded with Tf−HRP as indicated and exposed to DAB in the presence or absence of peroxide. LDM membranes were prepared and immunoblotted using anti-GLUT-3 antibodies to determine the extent of ablation on the intracellular content of each of the clones. The results of at least three independent experiments of this type are presented in Table 1. Note that also shown in this immunoblot are a range of protein loads for one sample to illustrate the linearity of the immunoblot signal, and thus validate the quantitation. The exposure time for each of the immunoblots is different, reflecting the different levels of recombinant GLUT-4 expression in the different mutants. In panel b, LDM membranes from TAG 3B1 were immunoblotted with either anti-TfR (upper panel) or anti-GLUT-3 antibodies (lower panel). Note that incubating cells at 4 °C with Tf−HRP did not result in significant ablation of the TfR from the LDM membranes. In contrast, incubating cells with Tf−HRP for 3 h at 37 °C resulted in a peroxide-dependent loss of TfR from LDM membranes. Under the same conditions, ablation of TAG 3B1 was much less pronounced.

1). This does not reflect mistargeting of LAG to an inappropriate compartment because (i) the buoyant density of LAG-containing membranes is similar to that of endogenous GLUT-4, (ii) insulin stimulates LAG translocation to an extent similar to that of endogenous GLUT-4 (*22*), and (iii) vesicle immunoprecipitation data have shown that LAG cofractionates with another marker for the intracellular GLUT-4 compartment, the aminopeptidase vp165 (D. R. Melvin and G. W. Gould, data not shown). However, definitive analysis of the LAG-containing compartment was

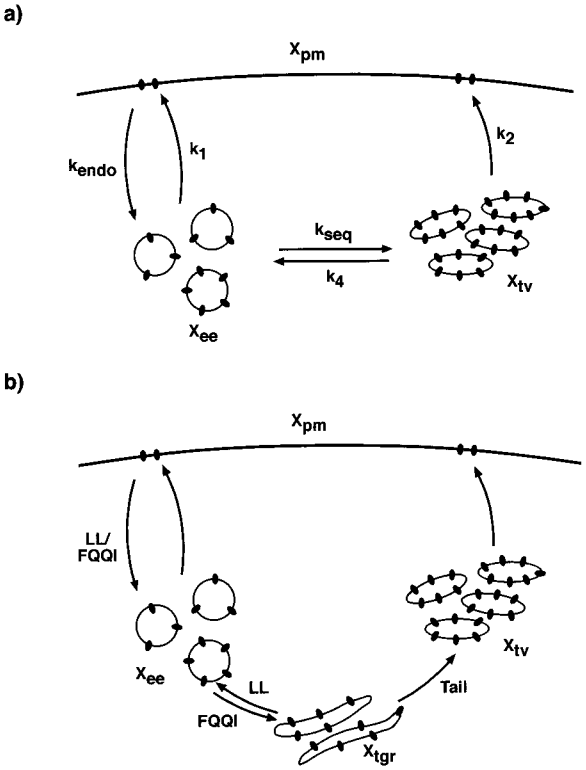

FIGURE 4: Schematic models for GLUT-4 trafficking in adipocytes. (a) A schematic model for GLUT-4 trafficking based on recent studies (*31*, *39*, *40*). In this model, GLUT-4 is internalized from the plasma membrane (X_{pm}) to a recycling compartment (X_{ee}) from which it is sequestered into a tubulovesicular storage compartment, which may be insulin-responsive (X_{tv}). The rate constants defining such steps are shown. (b) A refinement of the model in panel a, in which an extra compartment, the trans-Golgi network (X_{tgn}), is considered as an integral compartment in GLUT-4 recycling. Note that the sequestration of GLUT-4 into X_{tv} may arise from either the endosomal (X_{ee}) or TGN (X_{tgn}) compartments in this model.

not possible because of the poor efficiency of the anti-GLUT-3 antibodies to immunoadsorb LAG-containing vesicles. Hence, our contention that LAG is targeted to the same compartment as endogenous GLUT-4 has not been definitively demonstrated.

Interpretation of Mutant GLUT-4 Trafficking between Multiple Intracellular Compartments: A New Model for GLUT-4 Trafficking. We have attempted to interpret our data in light of existing models describing the intracellular distribution of GLUT-4 in adipocytes (*31*, *39*). The studies described herein imply that mutating either F^5 or L^{489} and L^{490} disrupts the intracellular distribution of GLUT-4 within the endosomal/TGN system. Models of GLUT-4 trafficking have suggested the presence of at least two intracellular GLUT-4 pools in adipocytes (Figure 4A; *31*, *39*). The first corresponds to a recycling endosomal compartment (X_{ee} in Figure 4a), which represents the compartment to which GLUT-4 is delivered upon internalization from the plasma membrane. The second pool is segregated from the recycling endosomal pool, and has been referred to as the tubulovesicular compartment (X_{tv} in Figure 4a). It has been suggested that this pool may regulate the cell surface levels of GLUT-4 in response to insulin (*31*, *39*). Using this model, we suggest that X_{ee} can be represented by the ablated compartment and X_{tv} by the nonablated compartment; X_{pm} is the plasma membrane pool.

Our analysis of both endogenous GLUT-4 (*33*) and TAG 3B1 (Table 1) by ablation suggests that the distribution of these proteins between X_{ee} and X_{tv} is roughly 40%:60%. Intracellular FAG exhibited an increased level of ablation compared to either TAG or wild-type GLUT-4, suggesting that this protein was not efficiently sorted into the nonablated GLUT-4 pool (X_{tv} in Figure 4a). In contrast, >90% of the LAG is in a nonablated compartment. If we invoke the model of Figure 4a, one of the consequences of the di-leucine mutation is a decrease in the X_{ee}/X_{tv} ratio, while this ratio is increased in the case of FAG. Thus, the distribution of these mutants in the context of the model of Figure 4a is defined by altering one or more of three rate constants, k_2, k_4, or k_{seq}. [Note that it can be shown that the ratio of X_{ee}/X_{tv} is independent of the internalization rate of GLUT-4 from X_{pm} into X_{ee} (*31*, *39*).]

The step defined by k_2 has been proposed to be independent of the protein cargo, and moreover has not been definitively established to occur in vivo. Furthermore, Birnbaum and Holman, when comparing trafficking of GLUT-4 and GLUT-1 in adipocytes (*31*, *40*), have argued that k_4 is independent of transporter isoform. Hence, to explain the distribution of FAG and LAG would require mainly an alteration of k_{seq}, which we felt was unlikely, especially as the mutation of LL results in a gain of function; i.e., LAG is more efficiently sorted out of X_{ee} relative to wild-type GLUT-4, suggesting it normally acts to limit the entry or retention of GLUT-4 in its storage compartment.

We felt it necessary therefore to consider an alternative model for GLUT-4 trafficking in adipocytes (shown in Figure 4b). Numerous morphological studies have shown that GLUT-4 is present in the TGN (*4*, *5*, *41*), a compartment not included in previous models. In our model, GLUT-4 is internalized into an endosomal compartment (X_{ee}). From this compartment, GLUT-4 is proposed to traffic to the TGN (X_{tgn}), and then into the tubulovesicular storage compartment (X_{tv}) (this compartment may also derive in part directly from the endosomal compartment). Addition of the TGN to the model of GLUT-4 trafficking provides an attractive explanation for the observed phenotypes of LAG and FAG without the need to invoke changes in k_{seq} or k_4.

We argue that internalization of GLUT-4 into the early endosomes (X_{ee}) is dependent upon the well-characterized internalization motifs, FQQI and LL (*42*, *43*). We suggest that GLUT-4 then is sorted into the TGN (X_{ee} to X_{tgn} in Figure 4b) in a step which may be dependent upon the FQQI motif in the amino terminus; this would account for the reduced level of entry of FAG into the nonablated compartment. In contrast, both TAG and LAG would be predicted to gain access to X_{tgn} in this model, and thus be able to sort to X_{tv}. The possibility that trafficking of GLUT-4 from X_{ee} to X_{tgn} is dependent upon the interaction of coat proteins with the FQQI motif is an attractive proposition. Recent studies have suggested that variants of the Tyr-X-X-hydrophobic sorting sequences may exist which dictate subtle sorting modifications in recycling membrane proteins (*26*, *44*); FQQI may be an example of this variation. If this motif were disrupted, then GLUT-4 would be expected to become localized to the recycling endosomal system. Our ablation data (Table 1 and Figure 3) are consistent with this phenotype for the FAG mutant.

In the case of endogenous GLUT-4, constitutive recycling back to the cell surface (k_1) would depend on movement of GLUT-4 from X_{tgn} to X_{ee}. On the basis of our ablation results, we suggest that this step might be dependent upon the interaction of adaptor proteins with the LL motif within the carboxy terminus. The sorting of some proteins at the TGN requires the formation of clathrin-coated vesicles, and is dependent upon the function of the AP-1 complex (*45*−*47*). We have shown that γ-adaptin (a component of the AP-1 complex) and GLUT-4 substantially colocalize within 3T3-L1 adipocytes (*48*). Thus, the sorting of GLUT-4 from X_{tgn} to X_{ee} might reasonably be proposed to occur via a di-leucine motif-dependent interaction with AP-1, as is known to occur in other membrane proteins (*49*−*51*). Thus, in the case of LAG, disruption of this motif would be predicted to result in its accumulation in either X_{tgn} or X_{tv}, as a consequence of a reduced rate of movement from X_{tgn} to X_{ee}.

On the basis of evidence from several laboratories, it can be shown that Tf−HRP does not gain access to the TGN. Previously, we and others have shown that no ablation of the TGN marker protein TGN38 occurs in adipocytes after Tf−HRP loading (*33*), and also in other cell types using electron microscopy (*52*). Hence, the observed decrease in the extent of ablation of LAG compared to either endogenous GLUT-4 or TAG could be a consequence of this protein being preferentially localized to X_{tgn} and/or X_{tv}.

The Endosomal Compartment Is Insulin-Responsive. We have argued that FAG is confined to the ablated compartment (X_{ee}). However, some comment on the overall phenotype of FAG is appropriate. Marsh and colleagues have shown that although the majority of FAG is localized to the surface of adipocytes, insulin is capable of eliciting an increase in cell surface FAG levels (*22*). Thus, it could be proposed that the major function of both the FQQI motif and the nonablated compartment may be to remove GLUT-4 from the recycling pool and thus maintain low cell surface levels in the absence of insulin. Although intracellular FAG is predominantly localized to the recycling pool, it still exhibits movement to the cell surface in response to insulin (*22*). This suggests that the recycling endosomal pool (X_{ee}) is itself "insulin-responsive", consistent with the insulin-stimulated translocation of other proteins that reside within endosomes, such as the TfR and GLUT-1 (*53*−*56*). For this reason, we do not refer to the nonablated compartment (X_{tv}) as the insulin-responsive compartment. Although this pool is probably mobilized to the plasma membrane in response to insulin, we emphasize that the recycling pool should equally be considered insulin-responsive.

SUMMARY

In this study, we have been able to resolve distinct effects of mutating either F^5 (FAG) or L^{489} and L^{490} (LAG) to alanines on the intracellular sorting of GLUT-4 in adipocytes. FAG was predominantly expressed at the cell surface, but the small intracellular pool exhibited a more restricted pattern of buoyant density than the endogenous GLUT-4. Furthermore, ablation analysis suggests that the intracellular population of FAG was extensively ablated in a fashion similar to that of other markers for the early endosomal compartment. We therefore suggest that mutation of F^5 to A results in the accumulation of GLUT-4 in an early endosomal compart-

ment, implying that this motif may be involved in trafficking GLUT-4 out of early endosomes. LAG exhibited a normal distribution by buoyant density analysis, but in marked contrast to FAG, ablation analysis indicated that LAG was expressed predominantly in a nonabated compartment. We propose that the di-leucine motif may be involved in the movement of GLUT-4 from the TGN to the recycling endosomal compartment. On the basis of these findings, we propose a new model for GLUT-4 trafficking in adipocytes which, although only speculative at present, provides a rational model with which to interpret GLUT-4 trafficking.

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