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Identification of further important residues within the Glut4 carboxy-terminal tail which regulate subcellular trafficking

Diane L. Cope[1], Shu-Hua Lee, Derek R. Melvin, Gwyn W. Gould*

Division of Biochemistry and Molecular Biology, Davidson Building, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

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Abstract The insulin-responsive glucose transporter, Glut4, exhibits a unique subcellular distribution such that in the absence of insulin >95% of the protein is stored within intracellular membranes. In response to insulin, Glut4 exhibits a large mobilisation to the plasma membrane. Studies of the amino acid motifs which regulate the unique trafficking of Glut4 have identified several key residues within the soluble cytoplasmic N- and C-terminal domains of Glut4. Of particular note is a Leu-498Leu-499 motif within the C-terminal domain that has been proposed to regulate both internalisation from the plasma membrane and sorting to an insulin-sensitive compartment. In this study, we have examined the role of the adjacent amino acids (Glu-491, Gln-492 and Glu-493) by their sequential replacement with Ala. Our results are consistent with the notion that Glu-491 and Glu-493 play an important role in the sub-endosomal trafficking of Glut4, as substitution of these residues with Ala results in increased levels of these proteins at the cell surface, reduced insulin-stimulated translocation and increased susceptibility to endosomal ablation. These residues, together with other identified sequences within the C-terminus of Glut4, are likely to be crucial targeting elements that regulate Glut4 subcellular distribution.

Key words: Glucose transporter; Endosome; Insulin; Adipocyte

1. Introduction

Insulin stimulates glucose transport in peripheral tissues by inducing the movement ('translocation') of a pool of intracellular Glut4-containing vesicles to the plasma membrane [1–3]. In the absence of insulin, Glut4 is distributed among elements of the endosomal system and the *trans*-Golgi network (TGN), and also in a system of tubulo-vesicular elements throughout the cytosol [4–9]. Studies from several laboratories have provided evidence in favour of a slowly recycling pool of Glut4 that is selectively mobilised to the plasma membrane in response to insulin (reviewed in [1,2]). Whether this represents a unique modification of the existing endosomal system or, as we and others have argued, a distinct population of 'secretory' vesicles (termed Glut4 storage vesicles, or GSVs) remains unclear. What is evident, however, is that Glut4 exhibits slow continuous recycling between intracellular compartment(s) and the plasma membrane [1–3].

Studies aimed at identifying the amino acid sequences that regulate the unique subcellular distribution of Glut4 have revealed the presence of two internalisation motifs within the cytosolic domains of Glut4: a Phe-5-based motif in the N-terminus and a Leu-498Leu-499 pair in the C-terminus [10–14]. Replacement of either of these motifs with alanine resulted in an accumulation of Glut4 at the plasma membrane and reduced rates of endocytosis of the expressed Glut4, providing good evidence that these motifs are involved in the internalisation of Glut4 from the cell surface [10–14]. However, there remains some debate regarding whether either or both of these regions are involved in the targeting of Glut4 to the slowly recycling compartment/GSVs. However, we and others have further suggested that these distinct motifs may also regulate the trafficking within the endosomal system, and have proposed that Phe-5 and Leu-498Leu-499 may regulate Glut4 trafficking either through the TGN or control flux through the slowly recycling pathway [12].

The presence of distinct sorting signals within membrane proteins has been shown to regulate their distribution within the endosomal system [15,16]. Many of these putative signals bind to coat proteins that are proposed to selectively mediate the transport of membrane proteins from one compartment to another [17,18]. Thus, the presence of multiple and distinct sorting signals within the cytosolic tails of proteins with complex trafficking itineraries is likely required to facilitate their interaction with different adapter protein sub-units at multiple sites throughout the cell. Moreover, the same sorting signal has been proposed to function at multiple points in the sorting itinerary (see for example [19]). Hence, further refinement of the precise signals involved in Glut4 trafficking is required. It has been suggested that the C-terminus of GLUT4 contains additional targeting information, based on analyses of chimeric transporter proteins expressed in insulin-responsive cells [11,14,20]. Consequently, we have examined the role of the C-terminus immediately distal to the LeuLeu motif in Glut4 trafficking in insulin-sensitive 3T3-L1 adipocytes.

In this ??study, we have examined the role of the adjacent amino acids immediately distal to the LeuLeu motif (Glu-491, Gln-492 and Glu-493) by their sequential replacement with alanine. Substitution of Glut-491 or Glu-493 resulted in increased levels of these proteins at the cell surface, reduced insulin-stimulated translocation and increased susceptibility to endosomal ablation. Mutation of Gln492 to alanine was without significant effect. These data suggest that residues adjacent to the LeuLeu motif may either regulate the function

*Corresponding author. Fax: (44)-141-330 4620.
E-mail: g.gould@bio.gla.ac.uk

[1] Present address: Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge, UK.

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of this motif, or constitute a hitherto unidentified motif involved in the subcellular trafficking of Glut4.

2. Materials and methods

2.1. Cell culture

3T3-L1 murine fibroblasts obtained from the American Type Culture Collection (Rockville, MD, USA). Fibroblasts were induced to differentiate 2 days after reaching confluence as described [9,11], except that insulin was used at 4 μg/ml. Cells were used between days 8 and 12 after induction of differentiation.

2.2. Antibodies

The anti-peptide polyclonal antibodies specific for either the 12 COOH-terminal residues of GLUT4 or the 14 COOH-terminal residues of human GLUT3 have been characterised and described elsewhere [11,21]. Dr Morris Birnbaum (University of Pennsylvania) generously provided the affinity-purified polyclonal rabbit antiserum generated against the cytosolic domain of the insulin-regulated aminopeptidase vp165/IRAP.

2.3. Construction of recombinant GLUT4 transporter cDNAs

The COOH-terminal epitope-tagged GLUT4 mutant was provided by Professor David E. James (University of Queensland) and has been previously described and characterised [11]. This mutant employed the C-terminal 14 amino acids of the human GLUT3 glucose transporter as an epitope tag, allowing its identification using an anti-Glut3 antibody. The indicated amino acid residues were mutated using the QuickChange kit (Stratagene, UK), and all mutants were completely sequenced prior to analysis.

2.4. Expression of GLUT4 mutants in 3T3-L1 adipocytes

cDNA constructs sub-cloned into the pcDNA3 vector (Invitrogen) were transfected into sub-confluent 3T3-L1 fibroblasts using the Calcium Phosphate Transfection kits (Invitrogen, UK) following the manufacturer's protocol. Neomycin-resistant colonies (0.8 mg/ml G418) were isolated and selected as described previously [11,22].

2.5. Differential centrifugation

Subcellular membrane fractions were prepared from basal and insulin-treated adipocytes by differential centrifugation using a protocol previously described in detail [11,23]. This protocol yields four membrane fractions designated as high density microsomes (HDM), low density microsomes (LDM), plasma membranes (PM) and mitochondria/nuclei (M/N). Here we have focused on the PM and LDM fractions because these fractions are enriched in cell surface markers and membranes encompassing intracellular GLUT4, respectively [11,23].

2.6. Preparation and use of HRP-conjugated transferrin

The transferrin-horseradish peroxidase (Tf-HRP) conjugate was prepared and used exactly as described previously [24]. Cells were used for ablation experiments 8–12 days post differentiation. Human apo-transferrin and all reagents for Tf-HRP synthesis were from Sigma (UK). ^{125}I-labelled transferrin and ^{125}I-labelled goat anti-rabbit antibodies were from Du Pont/NEN (UK). Cells were loaded with Tf-HRP for the times shown to allow the endosomal system to become loaded. Thereafter, cells were rapidly chilled, cell surface-attached Tf-HRP removed and the cells processed for diaminobenzidine (DAB) cytochemistry. In brief, duplicate plates were incubated with DAB and hydrogen peroxide was added to one of the plates to initiate a cross-linking reaction catalysed by the Tf-HRP in the endosomal compartment. The plate to which no peroxide was added serves as a control for comparative purposes. By comparison of cells incubated with or without peroxide, the localisation of proteins within the endosomal compartment can be determined (see [24] for full details).

Table 1
Sequence of the Glut4 C-terminus and the position of the mutated residues

Wild-type	…RTPSLL**EQE**VKPSTELEYLGPDEND
TAG	…RTPSLL**EQE**VKPSTELEYLGPDEND*MNSIEPAKETTTNV*
AQE	…------**A**----------
EAE	…-------**A**----------
EQA	…--------**A**----------

Shown is the sequence of the wild-type Glut4 C-terminus, the addition of the Glut3 epitope tag in italics (TAG) and the position of the three mutants examined in this study in bold.

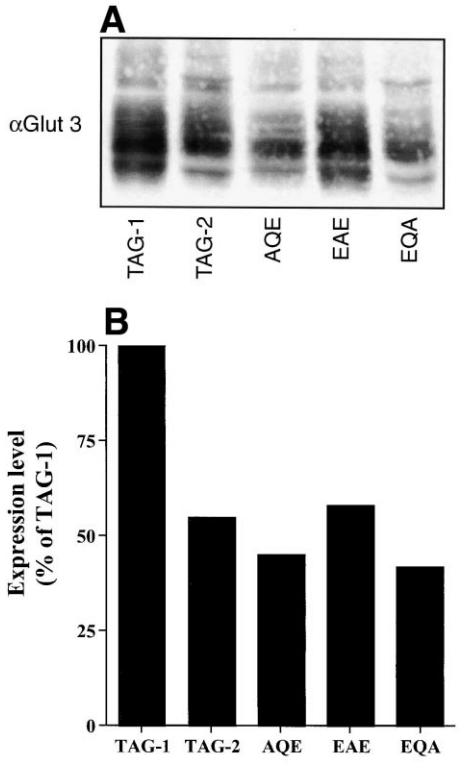

Fig. 1. Expression levels of the mutant Glut4 species. 25 μg of total membranes from clones expressing the indicated mutants were analysed by immunoblotting with anti-Glut3 antibodies to detect the recombinant protein. Shown in A are representative immunoblots from the clones employed in this study, which are quantified in B.

2.7. Electrophoresis and immunoblotting

Total cell membranes or subcellular membrane fractions (10 μg total protein) were subjected to SDS–PAGE using 7.5% or 10% polyacrylamide resolving gels and transferred to nitrocellulose. The protein concentrations of membrane fractions were determined using the bicinchoninic acid assay (Pierce, USA). Primary antibodies were detected by probing with either HRP-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies using the ECL system (Amersham, UK; Pierce, USA) or ^{125}I-labelled goat anti-rabbit IgG (Du-Pont/NEN, UK). Autoradiograms were quantified using a Bio-Rad densitometer.

2.8. Plasma membrane lawn assays for GLUT or IRAP translocation

After experimental manipulations, coverslips of adipocytes were rapidly washed in ice-cold buffer for the preparation of plasma membrane lawns exactly as described [25]. Triplicate coverslips were prepared at each experimental condition, and 5–10 random images of plasma membrane lawns collected from each in which plasma membrane lawns were stained with and-Glut3 (1:25) or anti-IRAP (1:250). These were quantified using MetaMorph (Universal Imaging, West Chester, PA, USA) software as described in [25].

3. Results

The sequence of the C-terminal cytosolic domain of Glut4 is shown in Table 1. We have generated three mutations within the region to produce mutant transporters designated AQE,

EAE or EQA as shown (Table 1). These mutants, together with wild-type epitope-tagged Glut4 (TAG), were expressed in 3T3-L1 adipocytes and stable clones selected. Fig. 1 compares the total levels of expression of epitope-tagged Glut4 in the cell lines chosen for analysis in this study. For each of the mutants, we have performed an analysis of the subcellular distribution of the mutant Glut4 in multiple clonal cell lines in order to avoid any clonal variability, but show here only the data relating to the clones shown in Fig. 1. Note that the level of expression of AQE, EAE and EQA in the clones

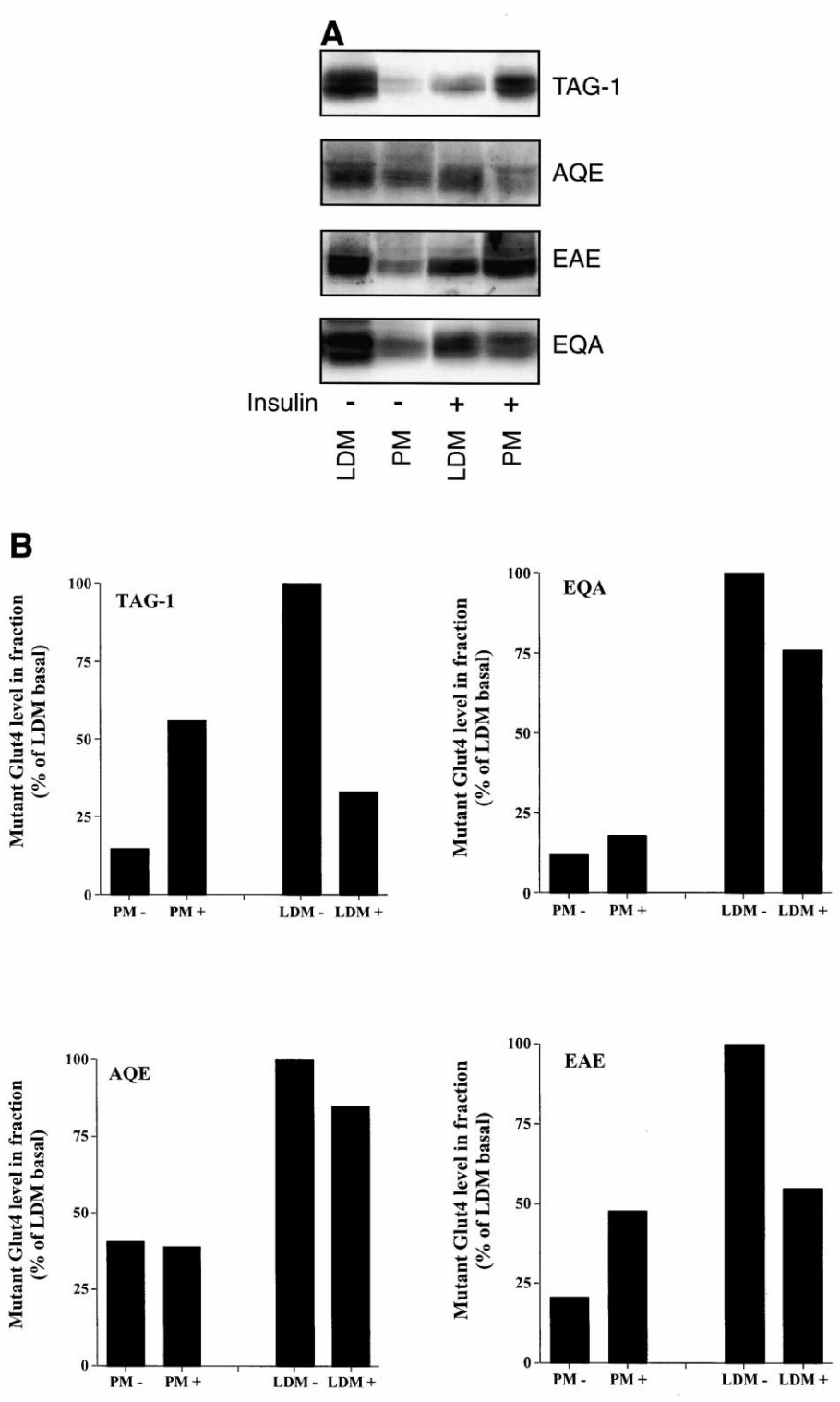

Fig. 2. Subcellular distribution of the mutant Glut4 species and the effect of insulin. Clones expressing the indicated mutant were stimulated with or without insulin (1 μM for 30 min) then subjected to subcellular fraction by differential centrifugation as outlined. 25 μg of plasma membrane or LDM fractions were analysed by immunoblotting with anti-Glut3 antibodies to detect the heterologously expressed mutant Glut4 species. Representative immunoblots are shown in A with quantification of three experiments of this type presented in B.

presented here is less than that of TAG-1. In all that follows, we will compare the mutant cell lines with that of TAG-1.

Fig. 2 shows the subcellular distribution of TAG-1 (the highest expressing TAG clone generated in this study) with representative clones of AQE, EAE and EQA. Fig. 2A shows representative immunoblots (quantified in Fig. 2B) in which anti-Glut3 antibodies were used to analyse the subcellular distribution and effects of insulin on the different mutant transporters. AQE characteristically exhibited a modest increase in plasma membrane levels of the Glut4 mutant in the absence of insulin compared to TAG-1 control cells (Fig. 2B). The plasma membrane/LDM ratios of the different mutants are presented in Table 2.

The ability of insulin to redistribute AQE and EQA to the plasma membrane was considerably impaired (Fig. 2A,B). By contrast, both TAG-1 and EAE exhibited striking translocation to the plasma membrane after insulin treatment. To further examine the ability of insulin to promote translocation of these species, we utilised plasma membrane lawn assays as a second independent assay of insulin-stimulated translocation, and compared the ability of insulin to stimulate mutant Glut4 translocation with the insulin-stimulated translocation of endogenous IRAP/vp165. The data are presented in Fig. 3 and further show that both AQE and EQA exhibit reduced insulin-stimulated translocation to the cell surface.

One possible explanation for the decreased translocation of AQE and EQA compared to TAG-1 is that they have failed to gain access to the proposed Glut4 storage vesicles. In order to address this, we performed endosome ablation analysis of these cell lines to estimate the extent of localisation of each of the mutants to the transferrin receptor-positive endosomal system. By loading cells with Tf-HRP and performing DAB cytochemistry ± hydrogen peroxide, we determined the extent of localisation of the different mutants to the recycling endosomal compartment [24]. The results of this analysis are presented in Fig. 4. Both AQE and EQA exhibit significantly greater susceptibility to Tf-HRP ablation than either TAG-1 or EAE, suggesting that a greater fraction of AQE and EQA populate the transferrin receptor-positive recycling endosomal system than is the case for TAG-1 or EAE.

4. Discussion

The molecular features that determine the unique intracellular distribution and insulin-responsive behaviour of Glut4 have been studied using a range of experimental systems. Although at least two regions of the protein have been implicated in both internalisation and sub-endosomal trafficking, the sequences that dictate the trafficking of Glut4 to the unique insulin-responsive compartment remain controversial

Table 2
PM/LDM ratio of the mutant proteins

	PM/LDM ratio
Glut4	0.16
TAG-1	0.15 ± 0.08
AQE	0.41 ± 0.10*
EAE	0.21 ± 0.06
EQA	0.12 ± 0.12

The PM/LDM ratio was determined from immunoblots such as those presented in Fig. 2 for basal (unstimulated) cells. The data for wild-type Glut4 were from [11,20]. *Significant increase compared to TAG-1 ($P < 0.05$).

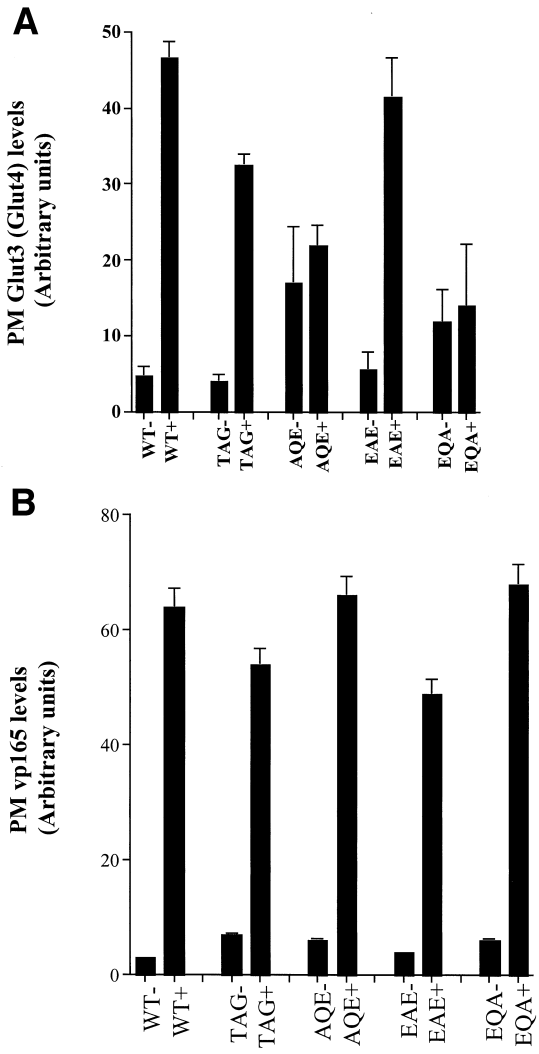

Fig. 3. Insulin-stimulated translocation of the mutant Glut4 species. As an independent assay of translocation, mutant Glut4 species were studied in plasma membrane lawns prepared from basal (−) or insulin-stimulated insulin (+) (1 μM for 15 min) cells and stained with anti-Glut3 or anti-vp165/IRAP. Shown are the data from a representative experiment, each column is the mean of the intensity of staining in 10 areas of three different coverslips, presented as mean ± S.D. The experiment was repeated with similar results. A: Anti-Glut3 results. B: Anti-vp165/IRAP results from the same experiment. PM Glut3 (Glut4) levels refers to the relative level of TAG Glut4 expressed at the cell surface under the conditions shown.

(see [11,14] for discussion of this point). Here, we have studied the role of three amino acids, Glu-491, Gln-492 and Glu-493, located adjacent to the LeuLeu internalisation motif in the Glut4 C-terminus. Mutation of Glu-491 and Glu-493 to alanine resulted in a marked decrease in the ability of insulin to promote translocation of these species to the plasma membrane. Moreover, these mutations rendered the expressed mutant Glut4 more susceptible to endosomal ablation, consistent with these mutants residing primarily in endosomal compartments. By contrast, mutation of Gln-492 to alanine had little effect on any parameter assayed in this study. At a minimum, these studies establish an important role for these residues in the subcellular trafficking of Glut4 in this cell type.

Based upon the studies reported here, it is not possible to

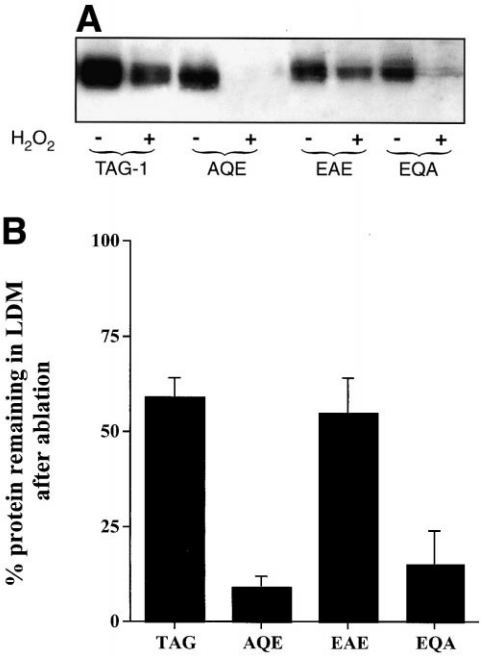

Fig. 4. Endosomal ablation of mutant Glut4 species. The distribution of the mutant Glut4 species within the endosomal system was studied by endosomal ablation. To this end, duplicate plates of cells were loaded with Tf-HRP to fully equilibrate the recycling endosomal compartment. Both plates were then incubated with DAB in the presence (test) or absence (control) of hydrogen peroxide. Peroxide acts as an electron donor which in the presence of HRP transfers an electron to the DAB and thus generates a highly reactive cross-linking species in the lumen of the endosomal compartment. After this treatment, cells were washed and LDM fractions prepared and immunoblotted for the presence of TAG Glut4. The extent of ablation was defined as the decrease in the immunoreactivity of anti-Glut3 between these conditions. A: Representative immunoblot with each of the cell lines studied. B: Quantification of three experiments of this type, in which the mean value of three separate experiments (±S.D.) is presented.

draw firm conclusions regarding the effect of the mutation on specific aspects of the Glut4 trafficking itinerary. It may be speculated that the Glu-491→Ala mutation may have exerted a modest effect on internalisation of the transporter, based upon an apparent increase in the PM/LDM ratio of this particular mutant. However, the inability of the Glut3 epitope tag to allow efficient immunoprecipitation has precluded a detailed analysis in this regard.

The fact that both the Glu-491→Ala and Glu-493→Ala mutants exhibit increased susceptibility to endosome ablation argues that an increased fraction of these mutants are colocalised with transferrin receptors than is the case for the endogenous protein (or TAG-1). This may reflect either a reduced rate of entry into the Glut4 storage vesicles, a reduced ability of the mutants to be retained in these vesicles, or alternatively could reflect a quite distinct pattern of intracellular trafficking of these mutants compared to endogenous Glut4. The inability of the Glut3 epitope tag to allow effective immunolocalisation has precluded detailed testing of these possibilities.

These caveats notwithstanding, our data are consistent with an important role of Glu-491 and Glu-493 in the intracellular trafficking itinerary of Glut4 in insulin-sensitive 3T3-L1 adipocytes. Whether these residues function in tandem with known motifs, such as the adjacent LeuLeu region [11,14] or the newly described targeting sequence located within TELEYLGP (residues 498–505) [20] remains to be determined. Given that this latter motif has been shown to play a key role in targeting to the Glut4 storage vesicles/insulin-responsive compartment, we favour a model in which Glu-491 and Glu-493 modulate presently undefined aspects of TGN/endosomal trafficking rather than directly controlling trafficking to the specialised compartment.

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