

RESEARCH ARTICLE

The first intracellular loop of GLUT4 contains a retention motif

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ABSTRACT

Glucose transporter GLUT4 (also known as SLC2A4) plays a major role in glucose homeostasis and is efficiently retained intracellularly in adipocytes and myocytes. To simplify the analysis of its retention, here, various intracellular GLUT4 domains were fused individually to reporter molecules. Of the four short cytoplasmic loops of GLUT4, only the first nine-residue-long loop conferred intracellular retention of truncated forms of the transferrin receptor and CD4 in adipocytes. In contrast, the same loop of GLUT1 was without effect. The reporter molecules to which the first loop of GLUT4 was fused localized, unlike GLUT4, to the trans-Golgi network (TGN), possibly explaining why these molecules did not respond to insulin. The retention induced by the GLUT4 loop was specific to adipocytes as it did not induce retention in preadipocytes. Of the SQWLGRKRA sequence that constitutes this loop, mutation of either the tryptophan or lysine residue abrogated reporter retention. Mutation of these residues individually into alanine residues in the full-length GLUT4 molecule resulted in a decreased retention for GLUT4-W105A. We conclude that the first intracellular loop of GLUT4 contains the retention motif WLGRK, in which W105 plays a prominent role.

KEY WORDS: GLUT4, Motif, Intracellular retention, Reporter, Chimera, Adipocytes, Insulin

INTRODUCTION

Glucose transporter GLUT4 (also known as SLC2A4) plays a crucial role in glucose homeostasis and is therefore an important target for the treatment of type 2 diabetes. GLUT4 mediates the uptake of glucose in muscle and fat. In the absence of insulin stimulation, GLUT4 is efficiently retained intracellularly. Upon insulin stimulation, GLUT4 translocates to the cell surface of myocytes and adipocytes where it transports glucose into these cells (Bogan, 2012; Govers, 2014). The combination of these two features, retention and insulin sensitivity, are highly unique to GLUT4. Although the intracellular trafficking of GLUT4 has been studied for decades, exactly how GLUT4 is retained intracellularly and how insulin impinges on this retention mechanism remains largely unknown.

The intracellular retention of GLUT4 is for a large part governed by the presence of GLUT4 in specialized GLUT4 vesicles, also known as GLUT4 storage vesicles (GSVs), that are devoid of endosomal markers. These GSVs are relatively static in the absence

of insulin (Fujita et al., 2010; Govers et al., 2004; Muretta et al., 2008). Upon insulin stimulation (and contraction in muscle), they move to the periphery of the cell, followed by their tethering, docking and fusion with the plasma membrane (Inoue et al., 2003; Martin et al., 2000; Patki et al., 2001; Xiong et al., 2010). Many proteins have been suggested to play a role in GSV-mediated retention of GLUT4, and include sortilin (Shi and Kandror, 2005), TUG (also known as ASPSCR1) (Bogan et al., 2012), AS160 (also known as TBC1D4) (Eguez et al., 2005) and SNARE(-regulating) proteins (Jewell et al., 2010).

In addition to GSVs, endosomes are also involved in intracellular GLUT4 retention. In cultured adipocytes, about half of the total cellular GLUT4 pool is present in endosomal compartments (Livingstone et al., 1996; Martin et al., 1996; Zeigerer et al., 2002). The same holds true for GLUT4 in muscle (Ploug et al., 1998; Ralston and Ploug, 1996). Given the fact that GLUT4 hardly recycles towards the plasma membrane in basal adipocytes (Govers et al., 2004; Karylowski et al., 2004), a retention mechanism must be present in endosomes. Another indication that GLUT4 can be retained intracellularly in endosomes comes from studies based on a mutant GLUT4 molecule in which the 12 C-terminal amino acid residues are replaced with those of GLUT3. When expressed in adipocytes, this molecule is exclusively present in endosomal compartments, but is still efficiently retained, albeit to a lesser extent when compared with wild-type GLUT4 (Govers et al., 2004). Finally, in fibroblast-like preadipocytes and CHO cells, GLUT4 is exclusively present in endosomes (Lampson et al., 2001; Zeigerer et al., 2002). Given that in these cells GLUT4 is retained intracellularly when compared with the transferrin receptor (Lampson et al., 2000; Zaarour et al., 2012), their endosomes must harbor a retention mechanism. Nonetheless, this GLUT4 retention is less efficient when compared with adipocytes, where about half of GLUT4 is present in non-endosomal compartment(s). Remarkably, the mechanisms implicated in the retention of GLUT4 in endosomes are completely unknown.

Given that the mechanisms implicated in GLUT4 retention are likely to be different in GSVs and endosomes, GLUT4 is likely to contain multiple motifs that are involved in its retention. These motifs might either direct the targeting of GLUT4 towards a retention compartment or be directly involved in the retention of GLUT4 within such a compartment. The C-terminal domain of GLUT4 contains sequences (e.g. T⁴⁹⁸ELEY⁵⁰²) that are somehow involved in intracellular GLUT4 retention as mutations within this domain largely prevent the targeting of GLUT4 into a non-endosomal compartment, presumably the GSVs, and increase cell surface GLUT4 levels (Blot and McGraw, 2008; Shewan et al., 2000). The N-terminal domain of GLUT4 also appears to contain a motif (F⁵QQI⁸) involved in the targeting of GLUT4 to a non-endosomal compartment (Blot and McGraw, 2008; Melvin et al., 1999). This motif is likely to affect GLUT4 retention at two distinct levels: it is probably involved in efficient internalization from the cell surface and in a targeting step within the cell (Blot and McGraw,

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2008; Garippa et al., 1994; Govers et al., 2004; Piper et al., 1993). Taken together, GLUT4 contains several targeting signals of which some might act, together or independently, on the same trafficking step. Alternatively, they might be implicated in distinct trafficking itineraries, but still have a common effect (for example inducing intracellular retention). Therefore, the identification of all the amino acid sequences within the full-length GLUT4 molecule that are involved in intracellular retention is likely to be a difficult task. We hypothesized that mutation of a single motif might not induce large changes in intracellular GLUT4 trafficking because other sequences within the GLUT4 molecule might still be capable of retaining GLUT4 within the cell. To circumvent this problem, we opted for another strategy, based on the use of reporter molecules.

Here, for the first time, the four small intracellular loops within the GLUT4 molecule were studied in regard to their capability to induce retention of reporter molecules in adipocytes. Truncated versions of the transferrin receptor (TfR) and CD4 were used as reporter molecules. Both TfR (Garrido et al., 2001; Johnson et al., 1998; White et al., 1998) and CD4 (Garrido et al., 2001; Le Maout et al., 2001; Mason et al., 2008; Rudell et al., 2014), lacking their cytoplasmic tails, have been used before as reporter molecules in order to identify motifs implicated in intracellular traffic. We present evidence that the first intracellular GLUT4 loop (i.e. the second intracellular domain, consisting of GLUT4 residues 103–111), contains a highly potent retention motif, capable of inducing retention of reporter molecules in adipocytes.

RESULTS

Intracellular retention in 3T3-L1 adipocytes

GLUT4 with an HA epitope tag within its first extracellular domain (Fig. 1) has been used extensively to study the intracellular localization of GLUT4 and is virtually absent from the plasma membrane in 3T3-L1 adipocytes (Fig. 2A, two left panels of second

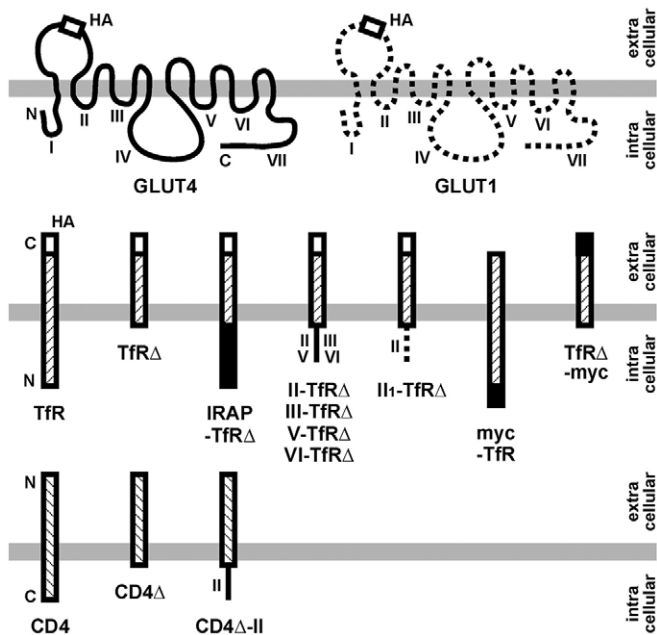


Fig. 1. Schematic representation of the molecules studied. All proteins, except for the CD4 molecules and some of the TfR molecules, contain an extracellular HA tag (white rectangle). GLUT4 structures are represented by solid lines, GLUT1 structures by dotted lines, IRAP and the Myc tag by black bars and reporter molecules by dashed bars. The gray bar represents the plasma membrane.

row). Given that GLUT4 can be recycling substantially via the plasma membrane even when cell surface levels remain relatively low (for example, in fibroblasts; Govers et al., 2004), experiments were performed in which living HA–GLUT4-expressing adipocytes were incubated for 10 min in the presence of high concentrations of anti-HA antibody, followed by fixation and permeabilization of the cells and detection of the anti-HA antibody. The adipocytes did not

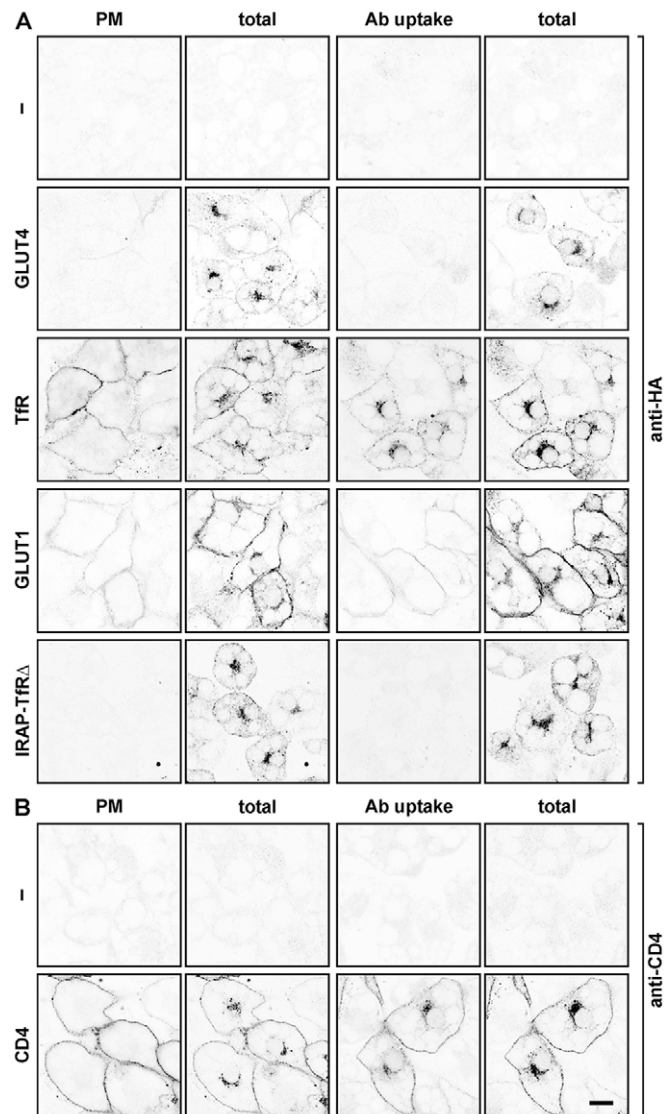


Fig. 2. Analysis of the presence of various proteins at the cell surface of adipocytes. 3T3-L1 adipocytes ectopically expressing the indicated proteins were fixed and stained by indirect fluorescent labeling using an anti-HA (A) or anti-CD4 antibody (B) in the absence of the membrane-permeabilizing agent saponin ('PM', first column). Both anti-HA and anti-CD4 antibodies label the proteins within their extracellular or luminal domain. Subsequently, the cells were permeabilized and stained for total cellular level of the protein using the same anti-HA and anti-CD4 antibodies and a secondary antibody conjugated to a different fluorophore ('total', second column). Alternatively, adipocytes were incubated for 10 min at 37°C in the presence of anti-HA (A) or anti-CD4 (B) antibody before fixation. Cells were permeabilized and the antibody that had been taken up was immunolabeled with fluorescent secondary antibody ('Ab uptake', third column). These cells were also stained for total cellular level of the expressed protein ('total', fourth column). The first rows in A and B show control adipocytes labeled with anti-HA and anti-CD4 antibody, respectively, followed by fluorescent secondary antibody, and show background labeling. Scale bar: 10 μm.

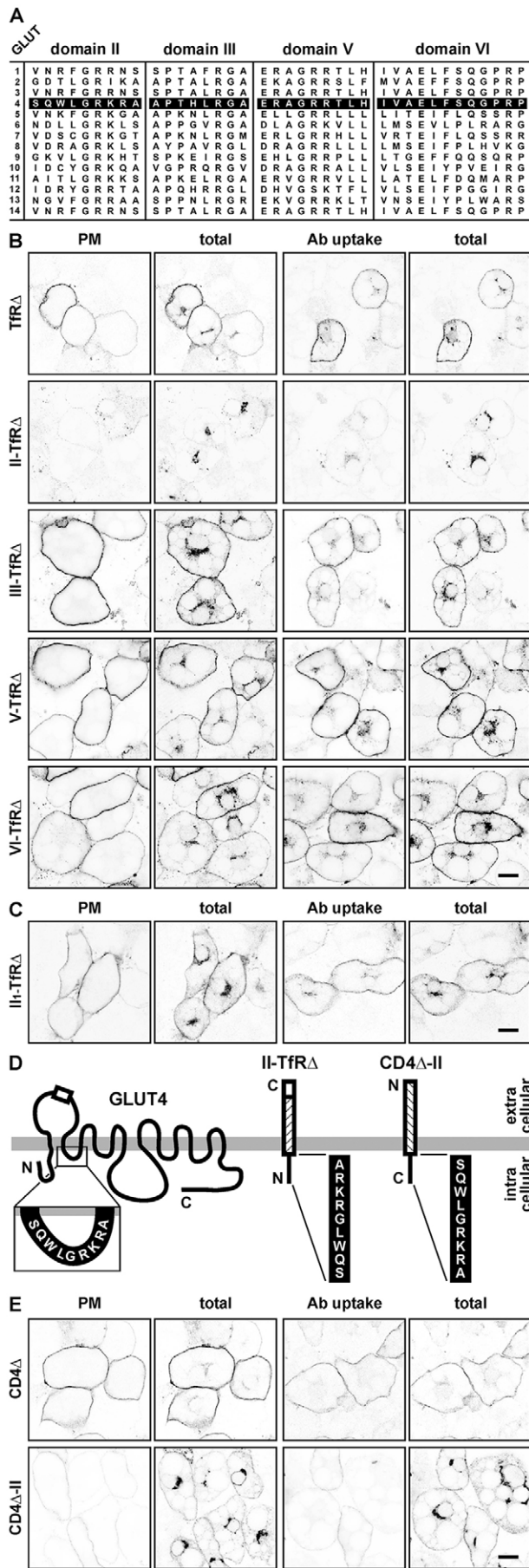


Fig. 3. Of the four short intracellular loops within the GLUT4 molecule, only the first one induces retention of the truncated reporter molecules TfR Δ and CD4 Δ . (A) Sequence alignment of the four short intracellular GLUT4 loops (domains II, III, V, and VI) of all members of the GLUT (SLC2A) family. Note that of all four domains, only domain II contains residues that are exclusive to GLUT4 (i.e. four out of nine residues). (B) 3T3-L1 adipocytes, expressing the short intracellular GLUT4 domains fused to the N-terminus of the truncated TfR reporter molecule TfR Δ (deprived of its cytosolic domain), were analyzed for the appearance of TfR Δ at the cell surface as described in the legend of Fig. 2. (C) Analysis of the effect of the second intracellular domain of GLUT1 (II₁) on the appearance of TfR Δ at the plasma membrane. (D) Schematic representation of full-length GLUT4 and the reporter molecules TfR Δ and CD4 Δ to which the second intracellular domain of GLUT4 (domain II) was fused. The orientation of domain II is highlighted for each of the three proteins. (E) 3T3-L1 adipocytes, expressing the truncated CD4 reporter molecule CD4 Δ (deprived of its cytosolic domain) or CD4 Δ fused to the second intracellular domain of GLUT4, were analyzed for the appearance of the reporter molecule at the cell surface using an anti-CD4 antibody. Scale bars: 10 μ m.

accumulate any substantial amount of anti-HA antibody at the cell surface or intracellularly (Fig. 2A, two right panels of second row), indicating that during these 10 min there was very little GLUT4 appearing at the cell surface. In sharp contrast, the constitutively recycling transferrin receptor (TfR) was present at the plasma membrane to a considerable extent and rapidly internalized antibody (Fig. 2A, third row). This demonstrates the efficient intracellular retention of GLUT4, when compared with TfR, in accordance with previous reports (Zaarour et al., 2012; Zeigerer et al., 2002). Compared with TfR, GLUT4 was also retained intracellularly, but to a lesser extent than GLUT4 (Fig. 2A, fourth row). Another molecule that is efficiently retained intracellularly in adipocytes is a chimeric molecule consisting of the cytoplasmic domain of IRAP (also known as LNPEP) fused to the transmembrane and extracellular domains of the transferrin receptor (IRAP-TfR Δ , also known as vpTR; Fig. 2A, fifth row). This molecule is known to behave similarly to full-length IRAP (and GLUT4) as far as its intracellular trafficking is concerned (Johnson et al., 1998; Subtil et al., 2000). CD4 was also included in our studies. This molecule did not need to be equipped with an epitope tag, as an antibody directed against the extracellular domain is readily available. Analyzing non-stimulated CD4-expressing adipocytes using this antibody demonstrated that CD4, like TfR, was constitutively trafficking towards the plasma membrane, in contrast to GLUT4 and IRAP-TfR Δ (Fig. 2B).

Effect of the four small intracellular domains of GLUT4 on intracellular retention of reporter molecules TfR and CD4

GLUT4 contains four small cytoplasmic loops (intracellular domains II, III, V, and VI; Fig. 1), of which only the first one is substantially different from the corresponding loop of the other glucose transporters (Fig. 3A). To determine whether these four domains contain a potential retention motif, these domains were fused to an HA-tagged truncated TfR, deprived of its cytoplasmic domain, and expressed in adipocytes (Fig. 3B). Visualizing the cell surface levels of the reporter and the chimeric proteins as well as the antibody taken up by living non-stimulated adipocytes revealed that the truncated TfR was constitutively trafficking towards the cell surface (Fig. 3B, first row, 'TfR Δ '). Fusion of the first cytoplasmic GLUT4 loop (domain II) largely changed the intracellular localization of the reporter molecule in that the reporter molecule became more localized in the perinuclear region of the cells and much less present at the plasma membrane (Fig. 3B, second row). Moreover, the adipocytes expressing this chimera took up much less

antibody from the extracellular milieu. Hence, this molecule resembled GLUT4 to a large extent, indicating that domain II confers intracellular retention to the reporter molecule. In contrast, the second, third and fourth short intracellular loops of GLUT4 (corresponding to intracellular domains III, V, and VI) did not induce retention of the reporter molecule. Remarkably, the first loop from GLUT1 (partially different from that of GLUT4; Fig. 3A) did not alter the intracellular distribution of the reporter molecule (Fig. 3C). In the experiments described above, the first GLUT4 loop was fused through its C-terminal residue to the truncated TfR reporter molecule. To determine whether the efficacy of this loop on intracellular retention was dependent on its orientation, we also fused this domain through its N-terminal residue to a truncated CD4 reporter molecule, lacking its cytoplasmic tail, hence inverting its orientation (Fig. 3D). Analysis of adipocytes expressing this reporter molecule and the reporter molecule fused to the first intracellular GLUT4 loop revealed that the GLUT4 domain also induced intracellular retention of the CD4 Δ reporter molecule (Fig. 3E). Fusion of this domain to the full-length versions of the TfR and CD4 molecules demonstrated that it imposed retention to full-length CD4 but not to full-length TfR (Fig. S1).

Domain II of GLUT4 imposes intracellular retention to reporter molecules without rendering them insulin sensitive

As the intracellular retention of GLUT4 is sensitive to insulin stimulation, we hypothesized that the retention of TfR Δ induced by the GLUT4 loop would also be insulin sensitive. Microscopical analysis of adipocytes incubated in the absence or presence of insulin demonstrated that this was not the case. The intracellularly retained chimeric molecule did not redistribute to the cell surface upon insulin stimulation and remained efficiently retained within the cell (Fig. 4A, two bottom rows), in contrast to GLUT4 (first two rows). A possible explanation for its lack of insulin sensitivity could be that the chimera might be retained within an intracellular compartment from where GLUT4 is not normally translocating. To address this possibility, we compared the localization of the chimeric protein with that of endogenous GLUT4 (Fig. 4B,C). This revealed that the intracellular localization of II-TfR Δ was largely distinct from that of GLUT4. To study the possibility that the chimeric protein might be insulin insensitive owing to intracellular retention within the biosynthetic route [i.e. the endoplasmic reticulum (ER) or Golgi] as a result of a defect in protein folding or conformation, experiments were designed in which the analysis of the intracellular traffic of proteins was limited to molecules that had already recycled via the plasma membrane (Fig. S2). This revealed that these molecules were efficiently retained as well, indicating that the second intracellular domain of GLUT4 induced intracellular retention of the reporter molecule in a post-Golgi compartment.

Quantification of cell surface levels and antibody uptake by chimeric TfR and CD4 molecules using 96-well plate assays confirmed that GLUT4 domain II induced intracellular retention of both reporter molecules (reducing cell surface levels and antibody uptake) and that this retention was not sensitive to insulin stimulation (Fig. 4D,E).

GLUT4 domain II induces retention of TfR Δ and CD4 Δ reporter molecules in the TGN

As the II-TfR Δ molecule was largely localized to GLUT4-negative compartments, we further examined where this chimeric molecule was located. Confocal immunofluorescence microscopy revealed that although GLUT4 substantially (but not perfectly) colocalized

with endocytosed transferrin and Myc-tagged transferrin receptor (in accordance with other studies; Livingstone et al., 1996; Zeigerer et al., 2002), both the TfR Δ -HA reporter molecule and the chimeric II-TfR Δ -HA molecule were largely excluded from Tf- and TfR-positive compartments (Fig. 5A,B). In addition, although GLUT4 was largely excluded from TGN38-positive structures (TGN38 is also known as TGOLN1) (Fig. 5C; Shewan et al., 2003), both TfR Δ -HA and II-TfR Δ -HA fully colocalized with TGN38 (Fig. 5C). The TGN localization of the tail-less TfR was quite surprising (and not published elsewhere as far as we know) as overexpressed full-length TfR as well as Tf internalized by endogenous TfR were largely excluded from TGN38-positive structures (Fig. 5D). Localization of the TfR Δ molecule was not likely to be affected by the HA tag as a Myc-tagged version of this molecule fully colocalized with the HA-tagged TfR Δ molecule (Fig. 5E). The co-expression of TfR Δ -Myc with II-TfR Δ -HA demonstrated that the fusion of the second intracellular domain of GLUT4 to TfR Δ did not alter the localization of the TfR Δ reporter molecule (Fig. 5E). In our cells, the cis and medial Golgi marker GM130 (also known as GOLGA2) colocalized neither with TGN38 nor with TfR Δ -Myc (Fig. 5F), providing further evidence for the localization of TfR Δ and II-TfR Δ in a post-Golgi compartment, in line with our other data (Fig. S2). In contrast to TfR Δ , the CD4 Δ reporter molecule substantially colocalized with endocytosed transferrin and endogenous GLUT4 (Fig. 6A,B), providing evidence for its presence in endosomal compartments. However, when fused to the second intracellular domain of GLUT4, the reporter molecule was largely excluded from Tf- and GLUT4-containing compartments (Fig. 6A,B) and colocalized, unlike CD4 Δ itself, with TGN38 (Fig. 6C). Taken together, these data show that domain II induces retention of reporter TfR Δ in the TGN without changing its intracellular location. Moreover, this domain induces retention of reporter CD4 Δ , while changing its intracellular localization from endosomes to the TGN.

GLUT4 domain II does not induce intracellular retention of reporter molecule TfR Δ in 3T3-L1 preadipocytes

The efficient intracellular retention of GLUT4 is highly specific for adipocytes and myocytes. Accordingly, GLUT4 retention is much more efficient in adipocytes than in fibroblastic preadipocytes (Govers et al., 2004; Zaarour et al., 2012; Zeigerer et al., 2002). To determine whether the retention of the reporter molecule induced by the first intracellular loop of GLUT4 is specific for adipocytes, as is the case for the retention of GLUT4, live preadipocytes were incubated with anti-HA antibody for 10 min. Visualization of captured antibody demonstrated that in the absence of insulin, preadipocytes readily took up antibody through GLUT4 as described before (Zaarour et al., 2012) and that the first loop of GLUT4 did not reduce the amount of antibody captured by the reporter molecule (Fig. 7), in contrast to the effect of this loop in adipocytes (Fig. 3). This indicated that the retention induced by domain II is specific for adipocytes as is the retention of full-length GLUT4.

Mutational analysis of domain II in reporter molecule TfR Δ and full-length GLUT4

To identify the amino acid residues within the nine-residue-long GLUT4 loop that are involved in intracellular retention, four GLUT4-TfR chimeric molecules were studied in adipocytes in which a pair of two subsequent residues of the GLUT4 sequence were changed en bloc into two alanine residues (Fig. 8A). The ninth residue of the GLUT4 loop was not taken into account in this

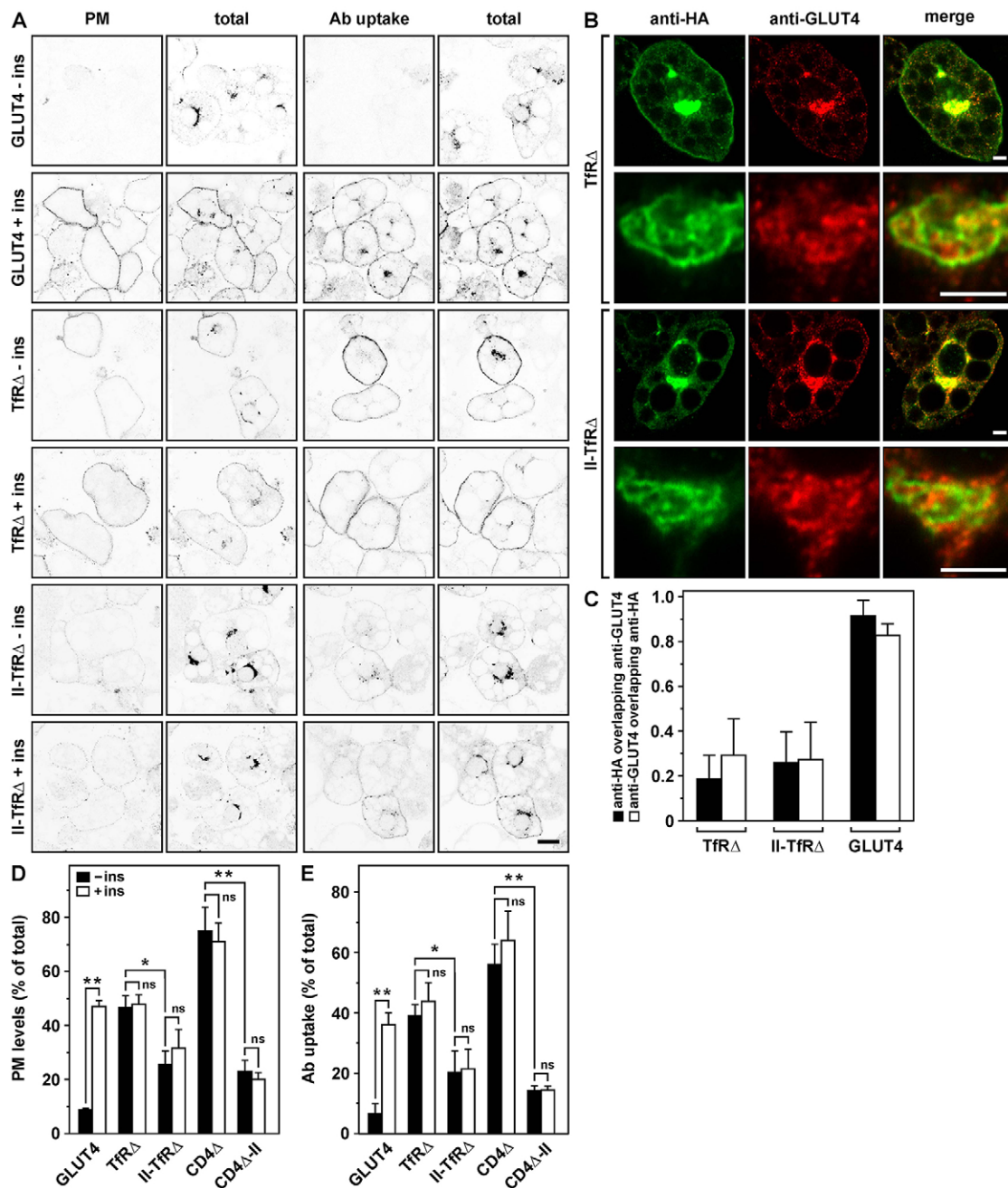


Fig. 4. The intracellularly retained chimeric proteins, consisting of the second intracellular GLUT4 domain fused to the reporter molecules, are not responsive to insulin stimulation. (A) 3T3-L1 adipocytes, ectopically expressing the indicated molecules, were incubated for 20 min in the absence or presence of 100 nM insulin and analyzed for the appearance of the reporter molecule at the cell surface as described in the legend of Fig. 2. Scale bar: 10 μ m. (B) Adipocytes expressing Tfr Δ or II-Tfr Δ were stained for the reporter molecule and endogenous GLUT4 using anti-HA and anti-GLUT4 antibodies, respectively. Scale bars: 3 μ m. (C) Data in B were analyzed using ImageJ software. Colocalization between the anti-HA and anti-GLUT4 signal was quantified for 3T3-L1 preadipocytes expressing HA-GLUT4 (two right bars; corresponding cell images are not shown). Results are mean \pm s.d. ($n=3$). (D,E) Adipocytes, expressing the indicated molecules were cultured in 96-well plates and incubated in the absence (black bars) or presence of 100 nM insulin (ins) (white bars). Cells were stained for cell surface levels (D) or subjected to 10 min of antibody uptake (anti-HA for GLUT4 and Tfr Δ molecules and anti-CD4 for CD4 Δ molecules) (E) as described in the legend of Fig. 2, except that total cellular (100%) levels were determined in separate wells as described in Materials and Methods. Fluorescence was measured using a fluorescence microtiter plate reader. Results are mean \pm s.d. ($n=3$). * $P<0.005$; ** $P<0.0005$; ns, not significantly different (unpaired t -test).

mutational analysis as this was already an alanine residue. This analysis revealed that of the sequence that constitutes the entire first intracellular GLUT4 loop (domain II), the WL and KR pairs (underlined residues in domain II: SQWLGRKRA) contributed to the retention induced by this domain, as mutation of these pairs individually markedly reduced the retention of the reporter-GLUT4

chimera (i.e. increased labeling at the plasma membrane; Fig. 8A, third and fifth row). Next, the four residues of these two pairs were mutated individually into alanine residues within the GLUT4-Tfr chimera II-Tfr Δ and in full-length GLUT4. Analysis of the II-Tfr Δ molecules in adipocytes (Fig. 8B) demonstrated that the tryptophan residue of the WL pair (W105 in GLUT4) and the lysine

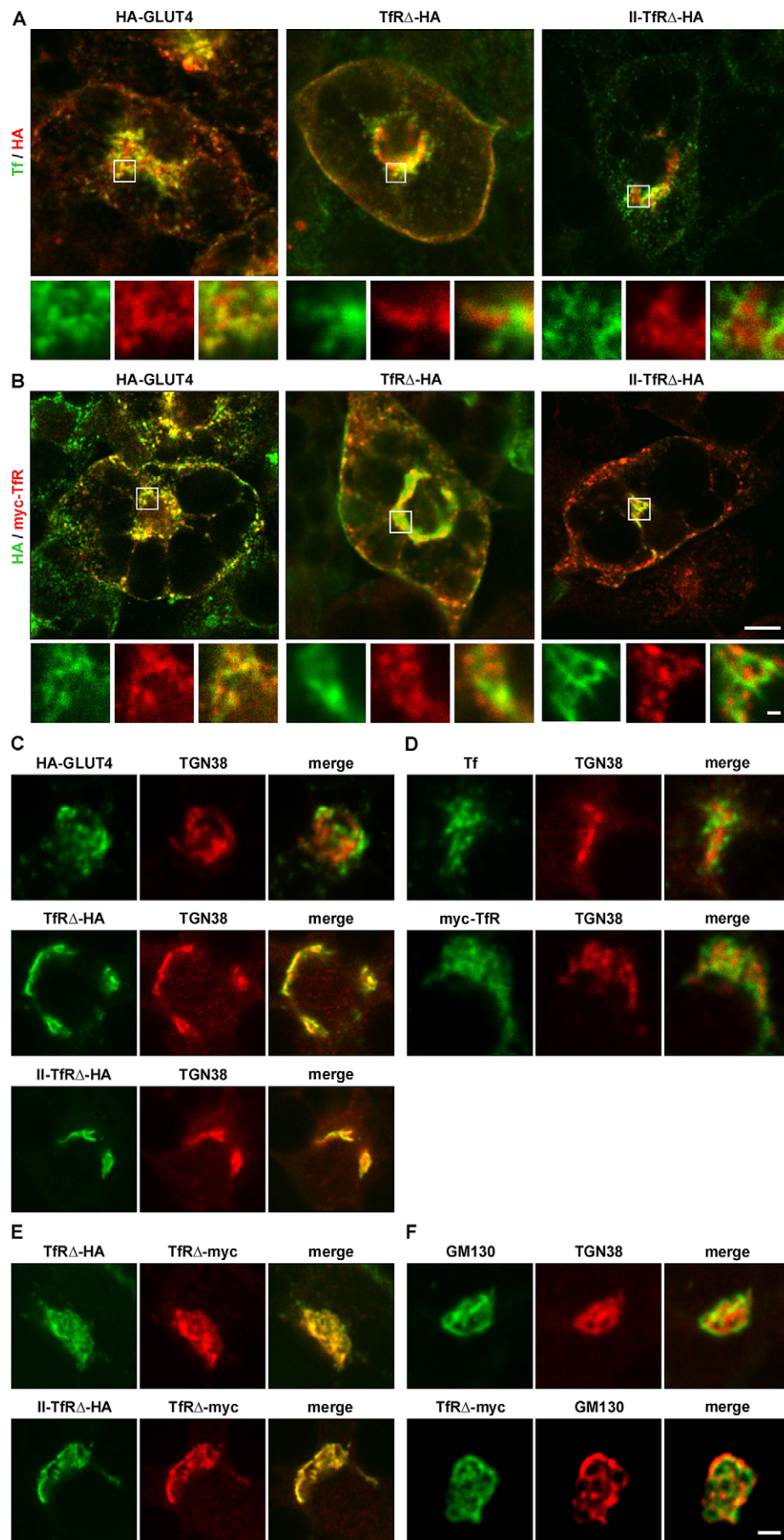


Fig. 5. Both the reporter molecule Tfr Δ and Tfr Δ fused to domain II of GLUT4 are localized to the TGN. (A) 3T3-L1 adipocytes, expressing HA-GLUT4, Tfr Δ -HA or II-Tfr Δ -HA were incubated for 1 h with 20 μ g/ml transferrin (Tf) and immunolabeled using anti-Tf and anti-HA antibodies. Part of the perinuclear region (white square) is enlarged in the three bottom panels. (B) Myc-tagged transferrin receptor (TfR) was co-expressed with the three HA-tagged proteins. Cells were immunolabeled using anti-HA and anti-Myc antibodies. Scale bars: 5 μ m (upper right image); 0.5 μ m (lower right image). (C) Adipocytes expressing the three HA-tagged proteins were labeled with anti-HA and anti-TGN38 antibodies. Only the perinuclear area of the cell is shown. (D) Adipocytes, expressing either exclusively endogenous TfR or Myc-tagged TfR, were immunolabeled for TGN38 and internalized Tf or for TGN38 and the Myc tag, respectively. (E) Myc-tagged Tfr Δ was co-expressed in adipocytes with either HA-tagged Tfr Δ or HA-tagged II-Tfr Δ . Adipocytes were immunolabeled using anti-HA and anti-Myc antibodies. (F) Myc-tagged Tfr Δ -expressing adipocytes were labeled either for cis- and medial-Golgi marker GM130 and TGN38, or for the Myc tag and GM130. Scale bar: 2 μ m.

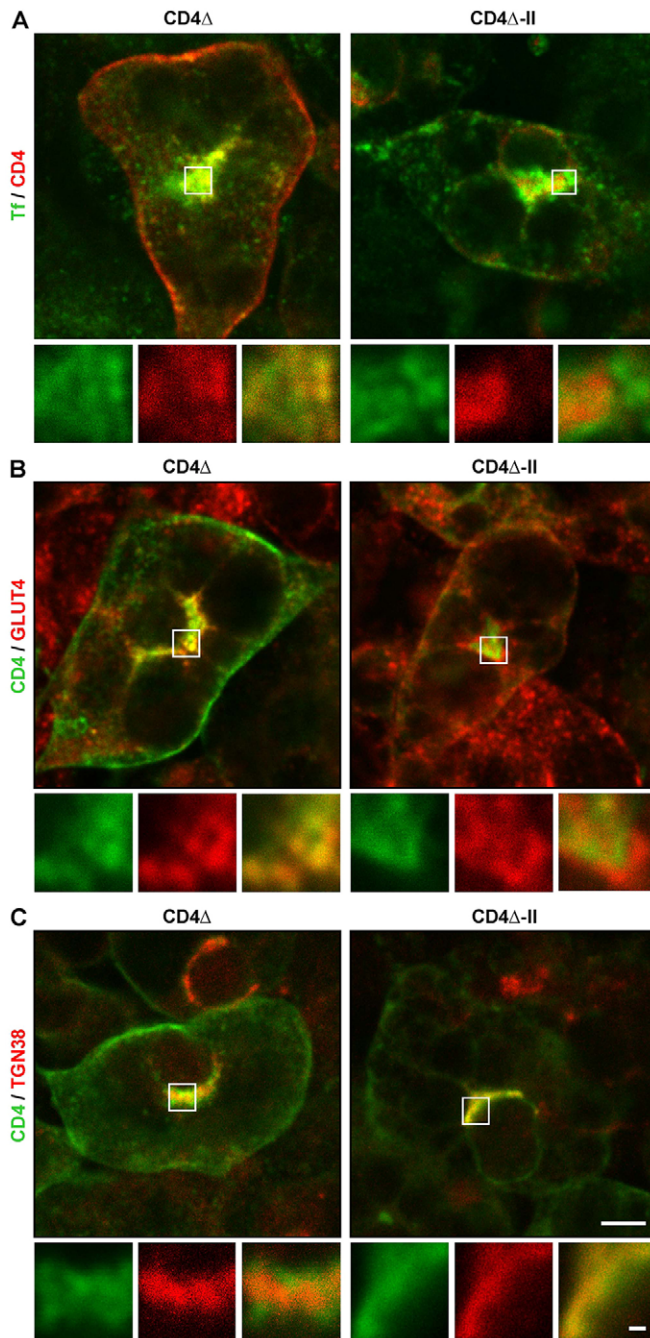


Fig. 6. The fusion of the second intracellular domain of GLUT4 to reporter molecule CD4 Δ induces the redistribution of the reporter molecule to the TGN. (A) 3T3-L1 adipocytes, expressing CD4 Δ or CD4 Δ -II were incubated for 1 h with Tf and immunolabeled using anti-Tf and anti-CD4 antibodies. Part of the perinuclear region (white square) is enlarged in the three bottom panels. (B,C) Adipocytes expressing the two CD4 Δ molecules were immunolabeled using anti-CD4 and anti-GLUT4 antibodies (B) or anti-CD4 and anti-TGN38 antibodies (C). Scale bars: 5 μ m (low magnification image); 0.5 μ m (high magnification image).

residue of the KR pair (K109 in GLUT4) were essential for the retention of TfR Δ induced by domain II, as both the tryptophan and lysine mutants were localized to a large extent at the plasma membrane (Fig. 8B, second and fourth row) in contrast to unmutated II-TfR Δ (upper row). Microscopical analysis of the mutant full-length GLUT4 molecules (Fig. 8C) revealed that

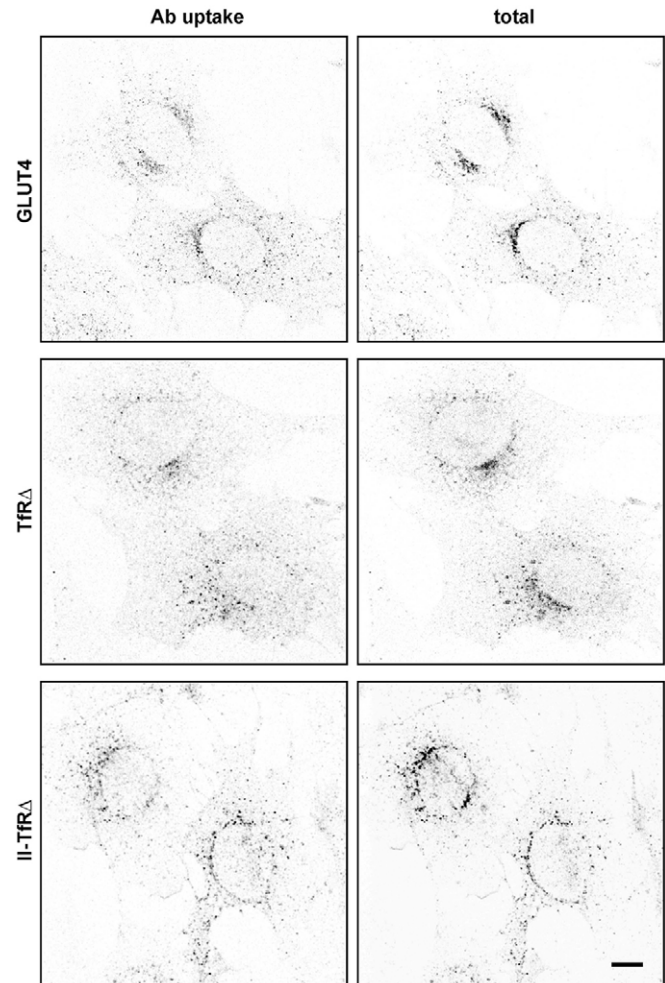


Fig. 7. The second intracellular domain of GLUT4 does not induce intracellular retention of the TfR reporter molecule in 3T3-L1 preadipocytes. Non-confluent preadipocytes, expressing the HA-tagged version of the indicated molecules were incubated for 10 min with anti-HA antibody and subsequently fixed and labeled for the antibody that had been taken up ('Ab uptake') as well as for the entire cellular pool of HA-tagged proteins ('total'). Scale bar: 10 μ m.

mutation of these four residues into alanine residues did not largely affect cell surface GLUT4 levels in basal adipocytes. Quantification of the cell surface levels of the reporter and full-length GLUT4 molecules not only confirmed that GLUT4 residues W105 and K109 (underlined residues in domain II: SQWLGRKRA) played an important role in the retention of the reporter molecule, but also that the W105A mutation marginally but significantly increased the presence of full-length GLUT4 at the plasma membrane, both in the absence as well as in the presence of insulin (Fig. 8D). The K109A and R110A mutations reduced the amount of GLUT4 at the plasma membrane, both in basal and insulin-stimulated adipocytes. The kinetics of translocation did not differ between these four mutants and wild-type GLUT4 (Fig. S3A). In addition, their internalization rates as well as the kinetics of their cell surface disappearance upon insulin withdrawal were identical to those of wild-type GLUT4 (Fig. S3B,C). To further analyze intracellular retention, antibody uptake experiments were performed (Fig. 8E). Incubation of adipocytes expressing these molecules in the continuous presence of anti-HA antibody confirmed that the W105A mutant was less efficiently retained in the absence of insulin stimulation, as these

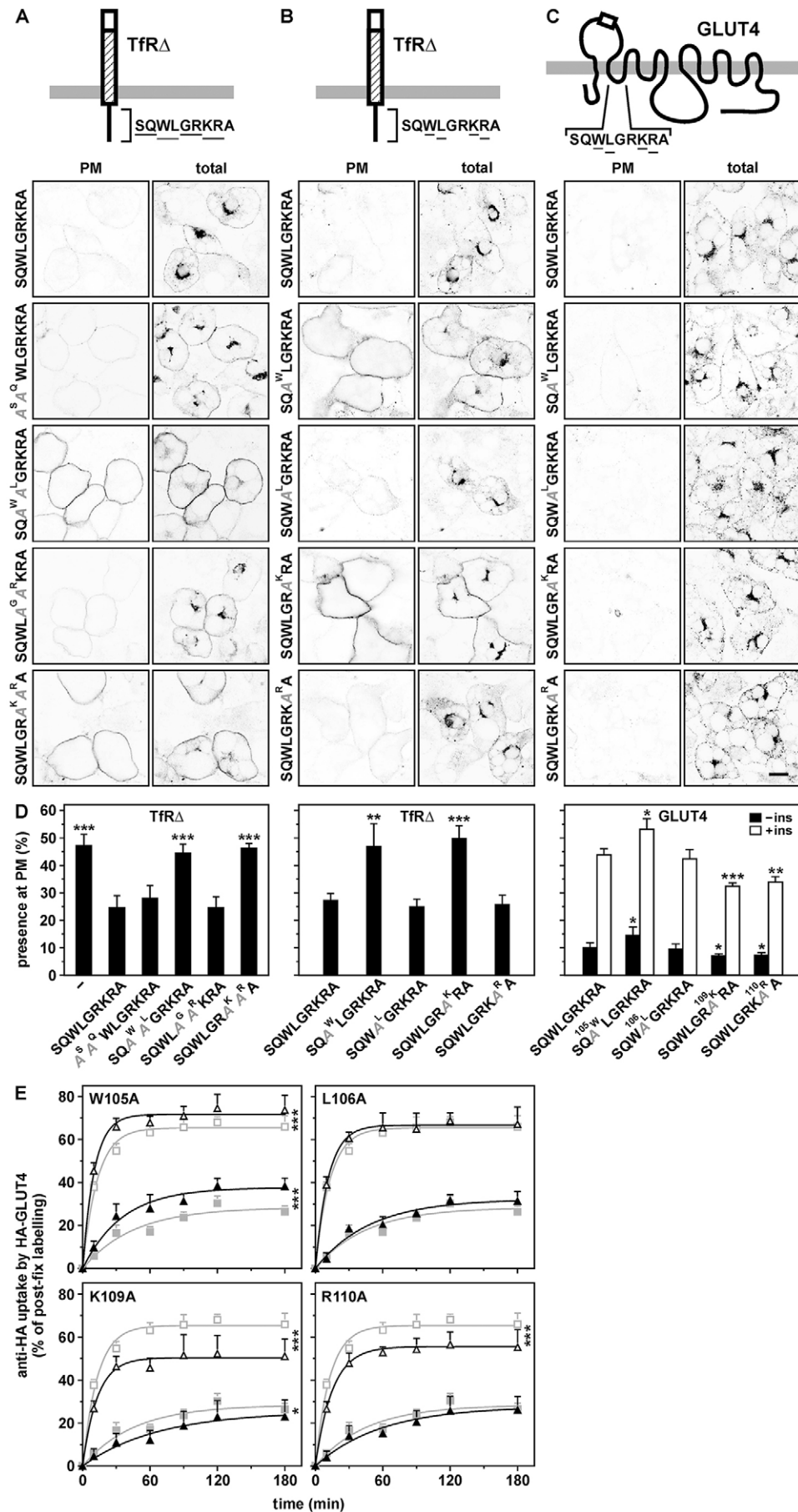


Fig. 8. Mutational analysis of the second intracellular GLUT4 domain in reporter molecule TFRΔ and in full-length GLUT4. (A) Pairs of GLUT4 residues were mutated into alanine residues within the GLUT4–TFR chimera II–TFRΔ. Cell surface levels of the chimeras were analyzed in 3T3-L1 adipocytes by fluorescence microscopy. The amino acid sequences on the left display the residues comprising the GLUT4 part of the chimera (wild-type sequence for the upper row and mutated sequences for the four rows beneath). Mutated residues are displayed in superscript, and introduced alanine residues in gray. In the schematic representation, the pairs of residues that were mutated are underlined. PM, plasma membrane. (B,C) Individual residues of the second intracellular domain were mutated to alanine residues within the GLUT4–TFR chimera II–TFRΔ (B) or in full-length HA-tagged GLUT4 (C). The presence of these molecules at the plasma membrane was visualized by fluorescence microscopy. Scale bar: 10 μm. (D) Cell surface levels of mutant GLUT4–TFR chimeras containing paired (left panel) or single mutations (middle panel), and of full-length GLUT4 mutants (right panel) in basal (black bars) and insulin-stimulated adipocytes (100 nM, white bars) were quantified as described in the legend of Fig. 4. The left graph corresponds to the mutants of A, the middle graph to the molecules of B and the right graph to the GLUT4 mutants of C. ins, insulin. Results are mean±s.d. (n=4). (E) Adipocytes expressing HA-tagged full-length wild-type GLUT4 (gray symbols and lines) or mutant GLUT4 (in black) were incubated for various time periods with saturating amounts of anti-HA antibody in the absence (closed symbols) or presence of 100 nM insulin (open symbols). Cells were fixed and labeled with fluorescent secondary antibody. Fluorescence was expressed as percentage of the amount of signal in control cells that were labeled with anti-HA antibody after fixation. Results are mean±s.d. (n=3). *P<0.05; **P<0.005; ***P<0.0005 versus controls: unmutated GLUT4 sequence (SQWLGRKRA) fused to TFR reporter molecule in left and middle panel of D, and wild-type GLUT4 in right panel of D and in E (unpaired t-test).

adipocytes took up more antibody than wild-type GLUT4-expressing adipocytes. In the presence of insulin, the cells that expressed this mutant also accumulated more antibody. While the L106A mutant was indistinguishable from wild-type GLUT4, both the K109A and R110A mutants took up less antibody in insulin-stimulated adipocytes. Moreover, the K109A-expressing adipocytes also accumulated less antibody in the absence of insulin, compared with wild-type GLUT4. Immunofluorescence microscopy did not reveal major differences in localization between wild-type GLUT4 and mutants W105A and K109A, when compared with the intracellular localization of IRAP and TGN38 (Fig. S4). Although the cell surface levels of the GLUT4-W105A mutant were somewhat increased when compared with wild-type GLUT4 (Fig. 8D), our studies on endosomal localization, using an endosomal ablation technique (Livingstone et al., 1996), did not reveal any differences between the two GLUT4 molecules [percentage of total intracellular protein ablated: 19.9 ± 7.6 for HA-GLUT4 and 25.1 ± 2.8 for HA-GLUT4-W105A (not significantly different); 67.8 ± 2.1 for endogenous TfR; 75.2 ± 3.4 for Tf-HRP; mean \pm s.d., $n=5$; data not shown].

DISCUSSION

GLUT4 is a complex molecule. This is mostly due to its 12 transmembrane domains, resulting in six extracellular and seven intracellular domains. Up to now, of these 13 extra- and intracellular domains, only three have been studied to a considerable extent: its first (largest) extracellular loop, containing its unique N-glycosylation site (Ing et al., 1996; Kim and Kandror, 2012; Zaarour et al., 2012) and its N- and C-termini, both of which are cytoplasmic (Blot and McGraw, 2008; Piper et al., 1993; Shewan et al., 2000). Here, we have demonstrated that the second nine-residue-long intracellular domain of GLUT4 induces intracellular retention when attached to two distinct reporter molecules. This domain, which is the first intracellular loop of GLUT4 (Fig. 1), consists of residues S¹⁰³QWLGRKRA¹¹¹. Within these chimeric molecules, domain II is not present as a loop but is either attached through its C-terminal alanine residue (for the TfRΔ reporter molecule) or through its N-terminal serine residue (for the CD4Δ reporter molecule). Hence, the retention induced by this domain is independent of whether it is attached through its N- or C-terminus nor does it require the attachment of both termini (as is the case in GLUT4). It is possible that this domain might just need to be positioned within close proximity of the membrane in order to induce intracellular retention. Maybe this is related to the potential interaction of domain II with membrane lipid phosphatidic acid (Heyward et al., 2008). Moreover, it could possibly explain why domain II induced retention of full-length CD4 (40 cytoplasmic residues) but not of full-length TfR (67 cytoplasmic residues), though this could also be due to interference from other motifs within the TfR cytoplasmic domain.

Remarkably, the same domain II of GLUT1 (sequence VNRFGRRNS) did not induce intracellular retention of the reporter molecule. A molecular analysis demonstrated that the duplets W¹⁰⁵L¹⁰⁶ and K¹⁰⁹R¹¹⁰ of GLUT4 domain II were required for retention of TfRΔ, as mutation of each pair individually into two alanine residues fully abrogated retention. Of these two duplets, W105 and K109 proved to be essential for retention of the reporter molecule, whereas L106 and R110 appeared not to play a major role. Mutation of these four residues individually into alanine residues within full-length GLUT4 revealed (1) that the mutation W105A increased cell surface GLUT4 levels in basal and insulin-stimulated adipocytes while increasing cell surface GLUT4

recycling, (2) that K109A and R110A mutants displayed a reduction in cell surface recycling (and reduced cell surface levels) in insulin-stimulated adipocytes and (3) that the L106A mutant did not differ from wild-type GLUT4. Of note, mutation of the four residues S¹⁰³QWL¹⁰⁶ en bloc into alanine residues reduced insulin-induced GLUT4 translocation by ~20% (Heyward et al., 2008 and our own results, data not shown). Mutation of such a relatively large sequence (i.e. almost half of the entire loop) might affect insulin sensitivity by destabilizing GLUT4 and inducing conformational changes in other parts of the molecule, independent of any direct effects on retention. Taken together, our analyses indicate that GLUT4 domain II, consisting of residues SQWLGRKRA, constitutes a retention motif by itself, capable of retaining reporter molecules within the cell, with major roles for W105 and K109 (underlined). The tryptophan residue of this motif also plays an important role in the retention of full-length GLUT4. Why mutation of K109 has distinct effects on the reporter molecule TfRΔ and full-length GLUT4 is unclear. In any case, our data clearly suggest that (the integrity of) domain II plays an important role in intracellular GLUT4 retention and that several individual changes within the primary structure of domain II affect intracellular GLUT4 traffic. The fact that domain II induces retention of reporter molecules in adipocytes but not in fibroblastic preadipocytes further corroborates its role in GLUT4 trafficking, as GLUT4 itself is also much more retained in adipocytes than in preadipocytes (Govers et al., 2004; Zeigerer et al., 2002).

Although domain II of GLUT4 induced intracellular retention of reporter molecules TfRΔ and CD4Δ, it did not render them insulin sensitive. This was not due to the retention of the chimeras within the biosynthetic route, as our studies have indicated that cell surface recycling reporter molecules were retained by domain II as well. However, it might be due to the localization of both chimeric molecules to the TGN (or a TGN38-positive TGN subcompartment) because GLUT4 itself is hardly present in this compartment under normal steady-state conditions. Remarkably, domain II retained the reporter molecule TfRΔ in the TGN without changing its localization, whereas it changed the steady state distribution of CD4Δ from endosomes to the TGN, which correlated with an increased retention of the reporter. It is possible that CD4Δ (devoid of any traffic signals) only transiently transits the TGN and that the fusion of domain II of GLUT4 to this reporter drastically slows down the exit of the reporter from the TGN, thereby inducing an accumulation of the reporter in this compartment. Given that domain II induces retention of both reporter molecules in the TGN, it is tempting to speculate that it might serve a similar function in GLUT4. GLUT4 might be temporarily retained in the TGN in order to be efficiently packaged into GSVs. This hypothesis appears to be in accordance with the data from Watson and colleagues who showed that, following biosynthesis, GLUT4 (and also IRAP) was efficiently retained intracellularly and did not acquire insulin responsiveness for the first 6–9 h, in contrast to GLUT1 and VSV-G that required only 2–3 h in order to appear at the plasma membrane (Watson et al., 2004). Possibly, newly synthesized GLUT4 is retained in the TGN before its entry into GSVs, explaining the delay in insulin responsiveness. Unfortunately, Watson and coworkers did not address this issue directly, but showed nonetheless that the insulin sensitivity of newly synthesized GLUT4 was further blocked by a dominant-interfering form of GGA. GGA is implicated in the exit of specific proteins from the TGN, away from proteins destined for the default constitutive exocytic pathway (Puertollano et al., 2001). This suggests that newly synthesized GLUT4 is retained in the TGN, awaiting its

GGA-dependent sorting into insulin-sensitive GSVs. In addition, following the insulin-induced release of GSVs and the subsequent return of the cells to the basal state, GLUT4 might be sorted via endosomes to the TGN, where it might be temporarily retained again, in order to be repackaged into GSVs. This mechanism might prevent GLUT4 from being constitutively exocytosed (i.e. taking the default TGN exit pathway) and explain why GLUT4 is absent from the TGN in both basal and insulin-stimulated cells at steady state. It might possibly also explain why domain II of GLUT4 does not induce retention of reporter TfRΔ in fibroblastic preadipocytes (Fig. 7). Given that in these cells GLUT4 is not packaged into GSVs (Zeigerer et al., 2002), there is no requirement for GLUT4 to be retained in the TGN.

How our data on full-length GLUT4 mutants, especially GLUT4-W105A, fit with this hypothesis is unclear at present, especially because this mutation did not seem to change the amount of GLUT4 in endosomes (see final part of Results section). In any case, retention of the reporter molecules in the TGN by domain II was not sufficient for their targeting into GSVs, as domain II failed to render them insulin sensitive, indicating that at least one other signal within the GLUT4 molecule was required for packaging into GSVs. Moreover, although domain II conferred intracellular retention to reporter molecules, these molecules were not as efficiently retained as wild-type GLUT4. This is obviously due to the presence of additional targeting motifs within GLUT4 that all contribute to the proper localization of GLUT4 as well as to efficient intracellular retention and insulin sensitivity. The presence of multiple targeting or retention motifs within GLUT4 also explains why mutations that largely reduced the retention of the chimeric molecule II-TfRΔ displayed relatively small effects on the retention of wild-type GLUT4 (Fig. 8). This is in line with studies that have reported that mutations in GLUT4 that dramatically changed its intracellular distribution, only marginally affected its intracellular retention (Blot and McGraw, 2008). Our data suggest that the three other short intracellular loops of GLUT4 do not contain any targeting motif. This is not very surprising as the primary structures of these three loops are largely conserved between the different members of the GLUT family, in contrast to the amino acid sequence of the second domain (Fig. 3A).

Taken together, we used an out-of-context approach to identify retention motifs within distinct GLUT4 domains, based on their fusion to reporter molecules and their expression in adipocytes. Such an approach permits the identification of targeting motifs that otherwise might not be found owing to redundancy of such motifs within the full-length wild-type GLUT4 molecule. Using this methodology, we discovered the existence of a retention motif within a small cytoplasmic loop. The discovery of GLUT4 retention motifs will be useful for the identification of elements of the retention machinery that undoubtedly interact with these motifs. Molecular insight into intracellular GLUT4 retention might contribute to the development of novel strategies to counterbalance hyperglycemia in type II diabetes.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes were obtained from the American Type Culture Collection ATCC/LGC Standards (Molsheim, France) and Platinum-E cells from Cell Biolabs Inc (San Diego, CA). Newborn and fetal bovine serum were from PAA (Les Mureaux, France). Media and HEPES were from Invitrogen (Cergy Pontoise, France). Insulin was from Lilly (Suresnes, France). Monoclonal anti-HA antibody (ascites; dilution for post-fix labeling 1:200) was from Covance (Emeryville, CA), monoclonal anti-CD4 antibody

(dilution for post-fix labeling 1:300) directed against the extracellular domain of CD4 was from Serotec (Kidlington, UK), polyclonal anti-IRAP antibody (1:100) was from Cell Signaling (Danvers, MA), chicken anti-Tf antibody (1:200) was from Sigma-Aldrich (Saint-Quentin Fallavier, France), monoclonal anti-GM130 antibody (1:400) was from BD Transduction Laboratories (Le Pont de Claix, France), polyclonal anti-Myc antibody (1:100) was from MBL International (Woburn, MA), monoclonal anti-Myc antibody 9E10 (1:600) was from Roche Life Sciences (Indianapolis, IN) and fluorescent secondary antibodies (1:200) were from Invitrogen (Cergy Pontoise, France). Polyclonal anti-GLUT4 antibody (1:100) was raised in rabbits (Zaarour et al., 2012). Polyclonal anti-TGN38 antibody (1:400) was kindly provided by Matthew Seaman (Cambridge Institute for Medical Research, UK). Restriction endonucleases NdeI and BglII were from Takara Clontech Laboratories (Mountain View, CA). BoxI and SgfI were from Fermentas Thermo Scientific (Pittsburgh, PA) and Promega (Madison, WI), respectively. All other restriction endonucleases and Phusion Taq polymerase were from New England Biolabs (Ipswich, MA). T4 DNA ligase was from Roche Life Sciences (Indianapolis, IN). Oligonucleotides were from Eurogentec (Seraing, Belgium). All other chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Black clear-bottom 96-well plates were from Greiner Bio-One (Courtaboeuf, France). Human CD4 cDNA was kindly provided by Jean Merot (Inserm UMR 1087/CNRS UMR 6291, Nantes University, France).

Molecular biology

For the retrovirus-mediated expression of proteins in 3T3-L1 preadipocytes and adipocytes, pBABE-puro and pBABE-neo vectors were used (Morgenstern and Land, 1990). The cDNA encoding HA-GLUT4 (GLUT4 with an HA epitope tag in its first luminal domain) inserted in pBABE-puro is described elsewhere (Govers et al., 2008), as well as the cDNA encoding GLUT1 with an HA tag in its first luminal domain between residues 56 and 57 (Berenguer et al., 2010). For the construction of the IRAP-TfR-HA chimera, the cDNA encoding IRAP residues 1–109 (cytosolic domain) was inserted by PCR into the MluI site (encoding threonine and arginine residues) of a pBABE plasmid containing the cDNA of TfRΔ (residues 60–760) followed by the HA-tag-containing EIDYPYDVPDYAE sequence immediately behind the MluI site. For the construction of TfR chimeras, the TfR cDNA was cloned into the BamHI/SalI restriction sites of pBABE-puro vector, with the insertion of a SgfI restriction site, SwaI site and Kozak GCCACC sequence between the BamHI site and start codon, and a MluI site (encoding TR residues) directly behind start codon. Before the stop codon, AscI, PmeI and PacI sites were inserted (encoding GAPFKPLIN residues) as well as the cDNA encoding the HA tag. These restriction sites were inserted for cloning purposes. The TfRΔ cDNA was identical to the TfR cDNA described above except that the cDNA encoding TfR residues 3 to 59 was removed (leaving eight residues of its cytoplasmic domain). For the construction of CD4 chimeras, the CD4 cDNA was cloned into the BamHI/SalI restriction sites of pBABE-puro vector, with the insertion of Kozak GCCACC sequence between BamHI site and start codon. Before the stop codon, a SgfI restriction site was inserted (encoding AIA residues). Between the stop codon and SalI site, SwaI and MluI restriction sites were inserted. These three restriction sites were inserted for cloning purposes. The CD4Δ cDNA was identical to the CD4 cDNA described above except that the cDNA encoding CD4 residues 428 to 458 was removed (leaving ten residues of its cytoplasmic domain). The pBABE-myc-TfR plasmid was constructed by PCR by insertion of the cDNA encoding the Myc tag AEEQKLISEEDL between the start codon and the third codon (encoding Asp3) of the human TfR cDNA. The pBABE-TfRΔ-myc plasmid was constructed by PCR by insertion of the cDNA encoding the Myc tag AEEQKLISEEDL between the final codon and the stop codon of the human TfRΔ (Δ3-59) cDNA. All pBABE plasmids were constructed by PCR using the oligonucleotides, PCR templates, cloning plasmids and restriction enzymes as indicated in Table S1. Where four oligonucleotides are indicated for a single cDNA construction, the splicing by overlap extension (SOEing) method was used. All parts of the plasmids that had been generated by PCR were verified by DNA sequencing (GATC Biotech, Cologne, Germany). The proteins encoded by most of these cDNAs are schematically represented in Fig. 1.

Cell culture

3T3-L1 preadipocytes were cultured and differentiated as described previously (Martinez et al., 2010). To express proteins ectopically in preadipocytes and adipocytes, preadipocytes were infected with retrovirus as described before (Zaarour et al., 2012). Platinum-E cells were used for the production of virus and transfected with the pBABE-puro vectors using X-tremeGENE (Roche Life Science; Indianapolis, IN). Cells were starved for 2 h in serum-free DMEM supplemented with 0.2% BSA before stimulation.

Analysis of the presence of proteins at the cell surface

The fluorescence-based assay for the detection of proteins at the cell surface has been described in detail elsewhere (Govers et al., 2008). Adipocytes expressing HA-tagged or CD4-chimeric proteins were cultured in 96-well plates, fixed and immunolabeled using saturating amounts of anti-HA or anti-CD4 antibody. Subsequently, the cells were labeled with fluorescent secondary antibodies after the cells had been incubated in the absence or presence of the permeabilizing agent saponin to label specific proteins at the cell surface or their total cellular pool, respectively. Plates were analyzed by a fluorescence microtitre plate reader (Fluostar Galaxy, BMG Labtechnologies, Offenburg, Germany). Cell surface levels were expressed as a percentage of total cellular levels.

For the analysis of cells cultured on coverslips, non-permeabilized cells were immunolabeled as described above to detect cell surface proteins (anti-HA or -CD4 antibody and Alexa-Fluor-488-conjugated anti-mouse-IgG labeling of non-permeabilized cells) and subsequently permeabilized and immunolabeled using the same primary antibody and an Alexa-Fluor-568-conjugated anti-mouse-IgG antibody (i.e. plasma membrane levels in green fluorescence, total cell levels in red fluorescence). Control cells expressing the same proteins were fixed, permeabilized and incubated with anti-HA or -CD4 antibody and Alexa-Fluor-488-conjugated anti-mouse-IgG antibodies, subsequently (i.e. total cell levels in green fluorescence). Labeled cells were imaged using a Leica SP5 resonant scanner multi-photon confocal microscope (Leica Microsystems, Wetzlar, Germany) in the department URMITE/CNRS-UMR6020 at the faculty of Medicine, Aix-Marseille University. First, for each expressed protein, the control cells (total cellular levels in green) were imaged and the PMT gain of the channel was set at a value at which only several pixels of the image became saturated (images not shown). Second, on the plasma membrane coverslip, cells were imaged that expressed correct amounts of the same labeled protein (total cellular levels in red fluorescence). Subsequently, those cells were also imaged for green fluorescence (plasma membrane levels in green) using the PMT gain determined from control cells. This permitted the imaging of relative cell surface levels.

Incubation of living cells with antibodies to measure intracellular retention

The continuous labeling of living cells with antibody marks the molecules that during the time of incubation appear at the cell surface (Govers et al., 2004; Zaarour et al., 2012). Adipocytes ectopically expressing molecules having a complete extracellular CD4 domain or bearing an HA tag within their extracellular domain were incubated either for 10 min or for various time periods (up to 3 h) with saturating amounts of anti-CD4 (3.3 µg/ml) or anti-HA antibody (12.5 µg/ml anti-HA), respectively. Cells were fixed, labeled with a fluorescent secondary antibody and analyzed by fluorescence microscopy using the same PMT gain as used to detect total cellular expression levels (as described above), or analyzed in a fluorescence microtitre plate reader. The amount of fluorescence was determined and expressed as percentage of the amount of label obtained from cells that had been immunolabeled after fixation with the same primary and secondary antibodies.

Measurement of colocalization and GLUT4 internalization by fluorescence microscopy

Colocalization between endogenous GLUT4 and either reporter molecule TfRΔ or TfRΔ fused to the second intracellular domain of GLUT4 (II-TfRΔ) was quantified using NIH ImageJ software. As a positive control, colocalization between anti-HA and anti-GLUT4 signal was quantified for 3T3-L1 preadipocytes expressing HA-GLUT4. Colocalization was

analyzed by Manders' overlap coefficient analysis in the ImageJ JACoP plugin. Coefficient M1 reflects the fraction of the anti-HA signal that overlaps the anti-GLUT4 signal. Coefficient M2 reflects the fraction of the anti-GLUT4 signal that overlaps the anti-HA signal. Coefficient values range from 0 to 1, corresponding to non-overlapping images and 100% colocalization images, respectively.

GLUT4 internalization was measured as described previously (Zaarour et al., 2012), based on protocols from others (Shigematsu et al., 2003; Williams et al., 2006; Williams and Pessin, 2008). HA-GLUT4-expressing adipocytes were incubated with insulin, cooled down on ice, washed extensively, and incubated on ice with anti-HA antibody. Cells were transferred to 37°C and allowed to internalize antibody for various time periods. The percentage of anti-HA-positive cells that displayed intracellular label was determined.

Statistics and curve fitting

All data are presented as means±s.d. Experiments were repeated at least three times. Representative experiments are shown. For fluorescence measurements in 96-well plates, four wells were used for each condition. General comparisons between data sets were evaluated using an unpaired two-tailed Student's *t*-test. Curves for the kinetics of continuous antibody uptake, cell surface translocation and cell surface disappearance after insulin withdrawal were calculated using one-phase exponential curve fitting and compared using an extra sum-of-squares *F* test (GraphPad Prism). Differences between data sets were considered statistically different when *P*<0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.T., M.B., and R.G. conceived and designed experiments. M.T., M.B., T.G., and R.G. performed experiments. M.T., M.B., T.G., M.C.A., M.P., F.P. and R.G. analysed the data. M.T., M.B., T.G., M.C.A., M.P., F.P. and R.G. wrote and reviewed the manuscript.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.183525/-/DC1>

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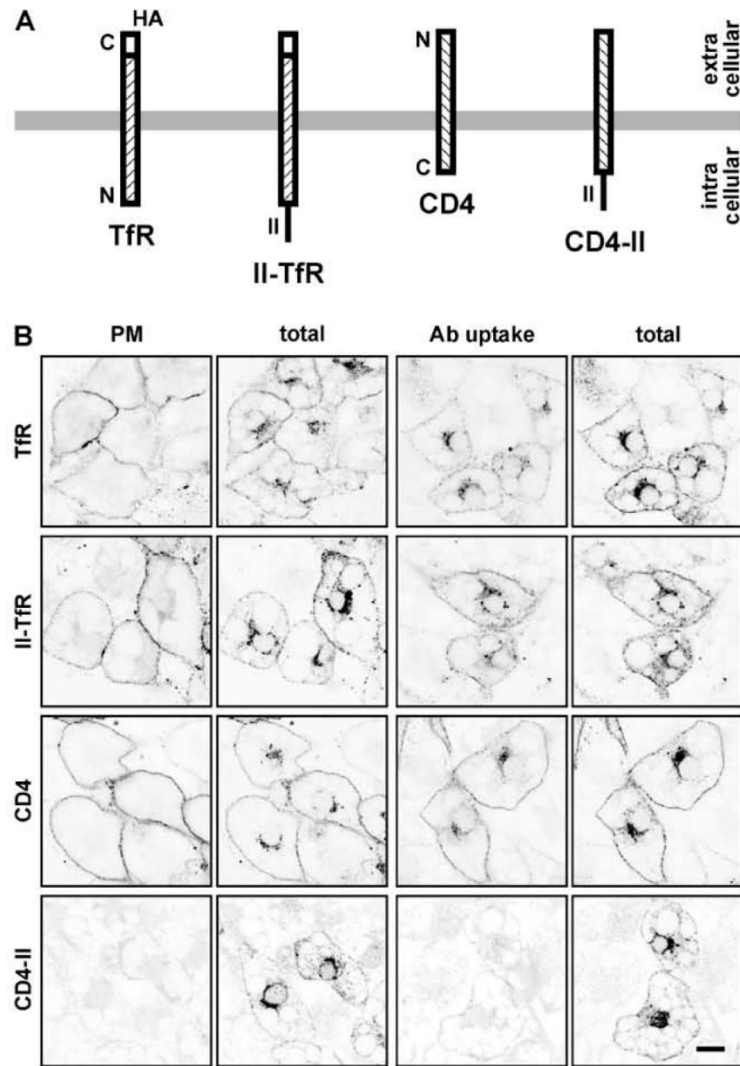


Figure S1. Domain II of GLUT4 induces intracellular retention of full-length CD4 but not of full-length Tfr. (A) Schematic representation of the molecules studied in this figure. (B) Plasma membrane levels and antibody uptake were studied for the full-length reporter molecules Tfr and CD4 and for both reporter molecules fused to the second intracellular domain of GLUT4, as described in the legend of Figure 2. Note that quantification of cell surface expression and antibody uptake of Tfr and II-Tfr using the 96 well plate assay demonstrated that domain II did not significantly change the amount of Tfr at the plasma membrane (25% decrease, not significantly different; data not shown). A quantitative analysis of the CD4-II molecule was not possible due to the very low expression level of this molecule. Bar, 10 μ m.

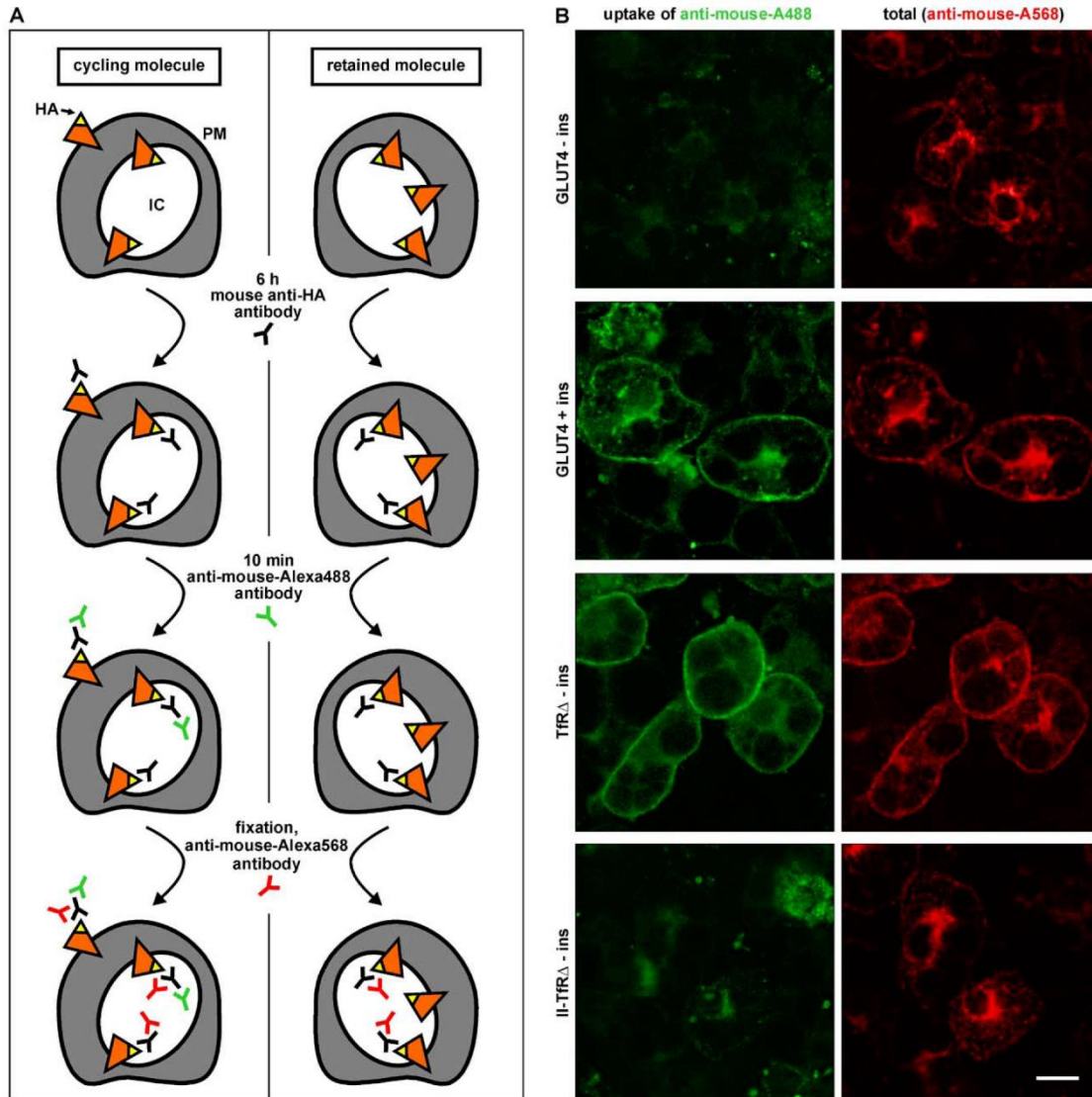


Figure S2. Domain II of GLUT4 induces retention of the reporter molecule in a post-Golgi compartment. 3T3-L1 adipocytes expressing HA-GLUT4, TfRΔ-HA, or II-TfRΔ-HA were incubated for 6 h with anti-HA antibody in serum-containing medium in order to label post-Golgi cell surface recycling molecules. After starving for 2 h in the absence of serum, the cells were incubated for 10 min with Alexa-488-conjugated fluorescent secondary antibody. This 10 min step resulted in the fluorescent labelling of the anti-HA antibody-bound molecule when it returned to the plasma membrane. Thus it allowed the distinction between molecules that, together with bound anti-HA, readily reappeared at the cell surface (within the 10 minute time-frame) and molecules that were retained intracellularly. After fixation, the cells were stained with Alexa-568-conjugated fluorescent secondary antibody in order to label all anti-HA antibody that had been taken up during the 6 h period. (A) Schematic representation of the outcome in case the protein studied is either a cell surface cycling molecule (left panel) or a molecule that is intracellularly retained in a post-Golgi compartment (right panel). IC, intracellular compartment. (B) Adipocytes were processed as described above and analyzed by fluorescence microscopy. As a control, HA-GLUT4-expressing adipocytes were also incubated with Alexa-488-conjugated anti-mouse antibody in the presence of 100 nM insulin as indicated in the figure.

Note that in case domain II induces intracellular retention principally within the ER/Golgi (biosynthetic route), the chimeric molecules (II-TfRΔ) that would reach the plasma membrane during the 6 h incubation (and thus labeled with anti-HA antibody) should take up the Alexa-488-conjugated secondary antibody during the 10 min incubation as efficiently as the TfR reporter molecule (TfRΔ). This was not the case, as the labelling pattern of the Alexa-488-conjugated anti-mouse antibody for TfRΔ and II-TfRΔ was largely distinct (two lower green panels). This approach demonstrated that of the proteins that had passed the ER/Golgi, both GLUT4 as well as the GLUT4-TfR chimera II-TfRΔ were retained intracellularly in the absence of insulin, indicating that domain II induces intracellular retention in a compartment beyond the biosynthetic route. The experiment was repeated three times, on different days and using different cell passages. One coverslip was used for each condition within each experiment. Four images were taken of each coverslip. All three experiments gave identical results. Representative images are shown. Bar, 10 μm.

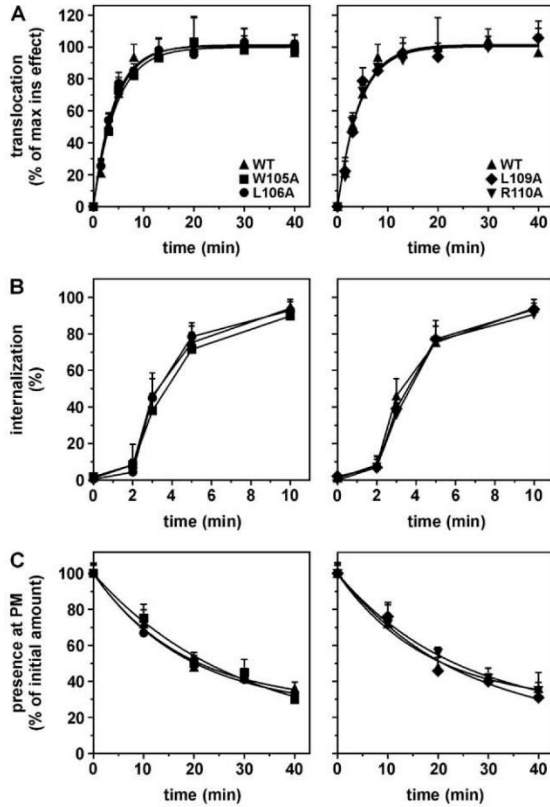


Figure S3. The mutation of individual residues within domain II of full-length GLUT4 does not change the kinetics of insulin-induced translocation, GLUT4 internalization, or the disappearance of GLUT4 from the plasma membrane upon insulin removal. (A) Adipocytes, expressing wild-type ('WT') or mutant GLUT4 molecules, were cultured in 96 well plates and stimulated with 100 nM insulin for various time periods. Cell surface GLUT4 levels were determined and expressed as percentage of the maximum increase in cell surface levels. All curves were calculated using exponential curve fitting and appeared not to be significantly different from WT. (B) Adipocytes were grown on coverslips, incubated for 20 min with 100 nM insulin to increase cell surface GLUT4 levels and cooled down on ice. Insulin was removed from its receptor by acid washes and cells were incubated with anti-HA antibody on ice to label GLUT4 at the cell surface. Coverslips were transferred to 37°C and incubated for various time periods before fixation. Anti-HA antibody was visualized using fluorescent secondary antibody and fluorescence microscopy. At each time point, the percentage of anti-HA-positive cells that displayed intracellular label was determined. (C) Adipocytes were incubated for 20 min with 50 nM insulin to increase cell surface GLUT4 levels. Cells were extensively washed in order to remove insulin and further incubated for different time periods in serum- and insulin-free medium, upon which cell surface GLUT4 levels were determined and expressed as percentage of the amount of GLUT4 that had translocated upon the initial insulin stimulation. All curves were calculated using exponential curve fitting and appeared not to be significantly different from WT. All experiments were repeated three times. Four wells were used for each condition within each experiment. Representative experiments are shown and display the mean \pm s.d.

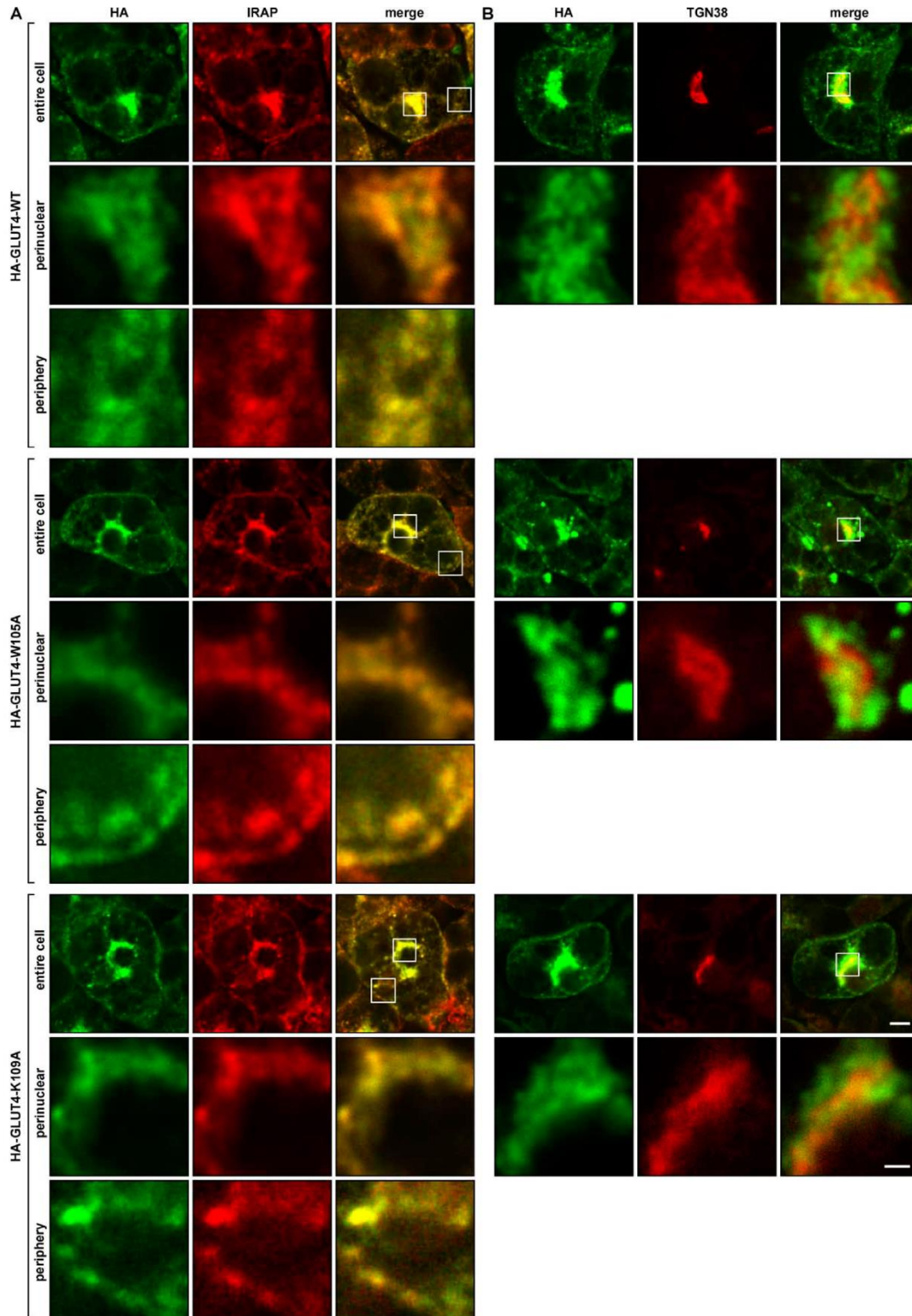


Figure S4. Mutation of Trp105 and Lys109 in full-length GLUT4 does not appear to alter its intracellular localization. 3T3-L1 adipocytes, expressing HA-GLUT4, HA-GLUT4-W105A or HA-GLUT4-K109A, were immunolabeled using (A) anti-HA and anti-IRAP antibodies, or (B) anti-HA and anti-TGN38 antibodies. First row of each GLUT4 molecule displays an entire cell. Parts of the perinuclear region and cell periphery (white squares) are enlarged in the second and third rows, respectively. All three GLUT4 molecules are perfectly colocalizing with IRAP in the perinuclear region of the cell and in the cell periphery. In contrast, they are minimally colocalizing (if at all) with TGN38. Bar in low magnification image represents 5 μ m, bar in high magnification image 1 μ m.

Table S1.

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