Trafficking of Glut4–Green Fluorescent Protein chimaeras in 3T3-L1 adipocytes suggests distinct internalization mechanisms regulating cell surface Glut4 levels

Kate A. POWELL^{*2,3}, Lachlan C. CAMPBELL^{*2}, Jeremy M. TAVARÉ[†], David P. LEADER^{*}, Jill A. WAKEFIELD^{*} and Gwyn W. GOULD^{*1}

^{*}Division of Biochemistry and Molecular Biology, Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K., and [†]Department of Biochemistry, University of Bristol, University Walk, Bristol BS8, U.K.

Insulin stimulates glucose transport in adipose and muscle tissue by stimulating the movement ('translocation') of an intracellular pool of glucose transporters (the Glut4 isoform) to the plasma membrane. We have engineered a series of chimaeras between Glut4 and green fluorescent protein (GFP) from *Aequoria victoria* and expressed these proteins in 3T3-L1 adipocytes by micro-injection of plasmid cDNA. In the absence of insulin, GFP–Glut4 is localized intracellularly within a perinuclear compartment and multiple intracellular punctate structures. In response to insulin, chimaeric GFP–Glut4 species exhibit a profound redistribution to the cell surface with kinetics comparable with the endogenous protein. The intracellular localization of GFP–Glut4 overlaps partially with compartments labelled with Texas Red transferrin, but is largely distinct from intracellular structures identified using Lysotracker-Red[®]. K⁺-depletion resulted in the accumu-

lation of GFP–Glut4 at the cell surface, but to an lesser extent than that observed in response to insulin. In contrast with native Glut4, removal of the insulin stimulus or treatment of insulin-stimulated cells with phosphatidylinositol 3'-kinase inhibitors did not result in re-internalization of the chimaeric GFP–Glut4 from the plasma membrane, suggesting that the recycling properties of this species differ from the native Glut4 molecule. We suggest that the recycling pathway utilized by GFP–Glut4 in the absence of insulin is distinct from that used to internalize GFP–Glut4 from the plasma membrane after withdrawal of the insulin stimulus, which may reflect distinct pathways for internalization of endogenous Glut4 in the presence or absence of insulin.

Key words: adipocyte, glucose transport, insulin.

INTRODUCTION

Insulin stimulates glucose transport in skeletal muscle and adipose tissue by virtue of the expression of the Glut4 glucose transporter isoform [1,2]. In the absence of insulin, this transporter is predominantly localized to intracellular membranes associated with the endosomal system, the *trans*-Golgi network (TGN) and within tubular vesicular elements throughout the cytosol [3–6]. In response to insulin, there is a rapid redistribution of Glut4 from these locations to the plasma membrane resulting in a dramatic increase in the rate of glucose transport into the cell [3–6]. Biochemical and microscopic studies have suggested that a significant proportion of the intracellular Glut4 is localized to a post-endosomal storage compartment, which is rapidly mobilized to the plasma membrane in response to insulin (reviewed in [1]).

Studies using transporter-specific photolabels have shown that Glut4 undergoes continuous recycling between the plasma membrane and intracellular membrane compartments in both the presence and absence of insulin [7–11]. This recycling has been shown to involve, at least in part, clathrin-coated pits [12,13] via a dynamin/amphiphysin-dependent mechanism [14], and is consistent with the observed localization of Glut4 to clathrin lattices in the plasma membrane and the partial overlap of Glut4 with the transferrin receptor (TfR) itinerary [15–17]. After insulin withdrawal, Glut4 is rapidly internalized from the plasma membrane and is effectively sequestered within the cell until such time as insulin is re-introduced [18–20]. It is not clear whether the

pathway/mechanism of internalization of Glut4 after insulin withdrawal is the same as that employed during the recycling of Glut4 in the absence of insulin.

Green fluorescent protein (GFP) [21,22] from the jellyfish *Aequoria victoria* has revolutionized cell biology, being widely used to 'tag' proteins of interest and to study the dynamics of their subcellular trafficking in living cells in real time [21]. Many membrane-membrane associated proteins have been studied using this method, including TGN38 [23], G-protein-coupled receptors [24,25] and proteins which utilize the secretory pathway [26], allowing detailed analysis of the dynamics of the behaviour of these proteins in a variety of different experimental circumstances. To that end, we have reported the use of GFP-tagged Glut4 expressed both in CHO cells and 3T3-L1 adipocytes [27,28]. In the present work, we have used a range of GFP–Glut4 chimaeras to study further the trafficking of Glut4 in adipocytes. We have shown that two chimaeras of GFP–Glut4 (GFP at either the N- or C-terminus) localize to intracellular membrane within 3T3-L1 adipocytes and exhibit insulin-dependent translocation to the plasma membrane with kinetics similar to that of the endogenous protein. These chimaeras exhibit limited overlap with TfR, appear to recycle between the plasma membrane and intracellular compartment(s) in the absence of insulin, and are localized to compartments distinct from both late endosomes and lysosomes. In contrast to native Glut4, removal of the insulin stimulus or treatment of insulin-stimulated cells with phosphatidylinositol 3'-kinase (PI3K) inhibitors did not result in

Abbreviations used: GFP, green fluorescent protein; Glut, glucose transporter; TfR, transferrin receptor; TGN, *trans*-Golgi network; PI3K, phosphatidylinositol 3'-kinase; DMEM, Dulbecco's modified Eagle's medium.

¹ To whom correspondence should be addressed (e-mail G.Gould@bio.gla.ac.uk).

² These authors contributed equally to this work.

³ Present address: Department of Biochemistry and Nutrition, Scottish Agricultural College, Auchincruive, Ayr KA6 5HW, Scotland, U.K.

re-internalization of either chimaera from the plasma membrane, suggesting that the recycling properties of GFP-tagged Glut4 differs from the native Glut4 molecule. We suggest that the recycling pathway utilized by GFR–Glut4 in the absence of insulin is distinct from that used to internalize GFP–Glut4 from the plasma membrane after withdrawal of the insulin stimulus, which may in turn represent distinct pathways for internalization of endogenous Glut4 in adipocytes.

MATERIALS AND METHODS

Materials

Wortmannin was from Sigma. Texas Red transferrin and Lysotracker-Red® were from Molecular Probes (Leiden, The Netherlands). LY294002 was generously provided by Dr Simon Cook (BBSRC, Babraham Institute, Cambridge, U.K.). All other reagents were as described previously [29,30].

Cell culture

3T3-L1 fibroblasts were grown and differentiated into adipocytes exactly as described previously [29,30]. Cells were used between passages 3 and 10 in all experiments, and between days 6 and 12 after induction of differentiation.

Glut4 mutants and GFP chimaera construction

Glut4–GFP chimaeras were generated using recombinant PCR from a triple mutant of GFP with enhanced fluorescent properties, as described in [21,23]. The chimaeras prepared were Glut4 with GFP fused at the N-terminus (GRP–Glut4), in which only the methionine at position 1 of Glut4 was absent from the original Glut4 primary sequence, or GFP at the C-terminus (Glut4–GFP) in which GFP was appended immediately after the extreme C-terminal Aspartate residue of Glut4. Glut4 containing the point mutations F⁵ > A (FAG–GFP) and LL^{489,490} > AA (LAG–GFP) were those described in [31–33], which were PCR-amplified and fused in frame with GFP, as described for the wild-type protein above. PCR reactions were performed using either Vent® or Taq polymerases and all products were completely sequenced on both strands to ensure fidelity of construction. Chimaeric cDNAs were sub-cloned into either pcDNA3 or pCR3.1 (Invitrogen), purified using Quiagen Maxi-prep kits and then further purified using Wizzard DNA clean-up columns (Promega, Southampton, U.K.). GFP–Glut1 was constructed by a recombinant PCR so that the GFP cDNA was attached to the N-terminus of the rat Glut1 cDNA, resulting in the deletion of the methionine residue at position 1 of the Glut1 primary sequence.

Cell microinjection

3T3-L1 adipocytes were injected at days 7–10 post-differentiation using an Eppendorf microinjector system fitted on to a Zeiss Axiovert microscope with a heated stage adjusted to provide a temperature of 37 °C in the bathing medium [Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal-bovine serum, penicillin/streptomycin and 20 mM Hepes, pH 7.4] which had been pre-equilibrated in a CO₂ incubator before use. For each coverslip, around 100 cells were injected into the nucleus with plasmid DNA diluted to a concentration of ~200 µg/ml in either distilled water or TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.4). Following injection, cells were washed twice with DMEM containing 10% (v/v) foetal bovine serum and incubated in DMEM containing foetal-bovine serum overnight in a CO₂ incubator at 37 °C.

Insulin stimulation experiments

Before experiments, coverslips of microinjected cells were incubated in serum-free DMEM in a CO₂-incubator for 2 h. After this time, either insulin (100 nM) or vehicle were added to duplicate plates and the incubation was continued for between 2 and 30 min in the incubator. Thereafter, cells were rapidly washed into KRP buffer (130 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 20 mM Hepes, 1.2 mM Na₂HPO₄, pH 7.4) containing 10 mM glucose at 37 °C, and viewed on a Zeiss Axiovert microscope as described below.

Insulin reversal experiments

For analysis of insulin reversal, the following experimental conditions were employed. After stimulation with insulin for 30 min as outlined above, cells were washed once with serum-free DMEM at 37 °C and then either: (i) incubated with anti-insulin antibodies in DMEM exactly as outlined in [28], or (ii) washed repeatedly in KRM buffer (as KRP, except that Hepes was replaced with 20 mM Mes, pH 6.0) on a hotplate at 37 °C for the times indicated in the Figure legends, prior to microscopic examination. In experiments where PI3K inhibitors were used, cells were washed in serum-free DMEM at 37 °C and incubated in serum-free DMEM containing either wortmannin (50–250 nM) or LY294002 (10 µM) in a CO₂ incubator for the times indicated. In order to ensure that these conditions effectively reversed insulin action, deoxy-Glc transport or Glut4 plasma-membrane lawn assays were performed at the same time on parallel plates of cells, using methods described previously [29,34].

K⁺-depletion experiments

Coverslips of cells expressing GFP–Glut4 were washed twice in either KRP or KRP in which the KCl had been replaced with NaCl, then incubated on a hotplate at 37 °C in 4 ml of the buffer for 2 h before image capture, which was performed in the same buffer.

Image capture and analysis

Injected cells, on coverslips, were imaged with a Zeiss 4 laser-scanning confocal microscope operating in confocal mode using either 40×, 63× or 100× Plan-APOCHROMAT 1.4 NA oil-immersion objectives. The samples were on a heated stage adjusted to provide a temperature of 37 °C in the bathing medium. Images were collected using either (or both) 488 nm or 543 nm lasers, with appropriate filter sets, for collection of the GFP signal (band-pass 505–520 nm), or 'red' signals (long-pass 590 nm). Data files were saved in .TIF format and analysed using MetaMorph software (Universal Imaging, West Chester, PA, U.S.A.). To quantify the extent of GFP–GLUT4 translocation to the plasma membrane, we employed MetaMorph software to isolate a region of interest, which corresponded to a defined area just above and just below the plane of the plasma membrane in a given confocal image. Fluorescence intensity in this area was expressed as a percentage of the total cellular GFP–Glut4 fluorescence. This analysis corrects for variations in cell morphology and for the expression level of GFP–Glut4 within individual cells and between separate experiments.

Use of fluorescently labelled markers for intracellular organelles

3T3-L1 adipocytes expressing GFP–Glut4 were incubated in the incubator with Texas Red transferrin (20 µg/ml) or Lysotracker-Red® (50 nM) in serum-free DMEM for 30 min at 37 °C. Thereafter, cells were washed in KRP/glucose, transferred to the

Figure 1 Expression of GFP–Glut4, Glut4–GFP and GFP–Glut1 in 3T3-L1 adipocytes and the effects of insulin

3T3-L1 adipocytes were microinjected with plasmid DNA encoding the GFP chimera and after a 24 h incubation in DMEM containing 10% (v/v) foetal-bovine serum, cells were gently washed in serum-free DMEM and incubated in a CO₂ incubator in serum-free DMEM for 2 h. Insulin (1 μ M) or vehicle were added directly to the DMEM, and at times from 5 to 60 min the microinjected cells were quickly washed into KRP buffer containing 10 mM glucose and imaged

microscope stage heated at 37 °C, and images were collected as described above.

Immunofluorescence analysis of endogenous Glut4

Endogenous Glut4 was localized in acetone-fixed cells using an antibody against the C-terminus of Glut4 as described previously [29].

Statistical analysis

Statistical analysis was performed using StatView 4 (Abacus, Berkeley, CA, U.S.A.).

RESULTS AND DISCUSSION

Insulin stimulates glucose disposal in peripheral tissues by virtue of the expression of the Glut4 isoform [1,2,35]. In the absence of insulin, this transporter is intracellularly sequestered within the elements of the endosomal system, the *trans*-Golgi network and a specialized storage compartment [3–6]. Glut4 is redistributed from these intracellular locations to the plasma membrane in response to insulin [3–6]. This paradigm of regulated membrane trafficking has been investigated using a range of methods, including transporter-specific photolabels, which have established that Glut4 undergoes continuous recycling between the plasma membrane and intracellular locations, even in the presence of insulin [7–11,36,37]. Upon removal of the insulin signal, Glut4 is effectively sequestered within the cell and leaves the plasma membrane with a half-time of about 6 to 7 min. However, the mechanism(s) of this trafficking event remain poorly understood.

With the aim of addressing this, in the present work, two chimaeric species between Glut4 and GFP were engineered. In the first, GFP was fused, in frame, at the N-terminus of Glut4 (termed GFP–Glut4) and, in the second, GFP was fused, in frame, at the C-terminus of the protein (termed Glut4–GFP). These cDNAs were expressed in fully differentiated 3T3-L1 adipocytes by microinjection and exhibited similar patterns of intracellular expression in the absence of insulin (Figure 1). Fluorescence was observed in a perinuclear compartment and also in multiple punctate structures throughout the cytosol in a pattern reminiscent of the native Glut4 identified in these cells. In response to insulin, both of these species exhibited extensive redistribution to the plasma membrane, resulting in a continuous ring of fluorescence around the perimeter of the cell and translocation of both GFP–Glut4 and Glut4–GFP to the plasma membrane in response to insulin was consistently observed (Figure 1, upper panel). This translocation was rapid; maximal redistribution was achieved within 15 min of exposure to insulin (results not shown), which is consistent with studies of endogenous Glut4 protein in these cells [38]. Under these conditions, we routinely observed insulin-stimulated translocation in > 95% of cells expressing GFP-tagged Glut4.

by confocal microscopy using the 488 nm laser for excitation of the GFP. Top panel: representative cells expressing GFP–Glut4 (top), Glut4–GFP (middle) or GFP–Glut1 (bottom) incubated with or without insulin for 30 min. Lower panel: quantification of several similar experiments to determine the fold increase in plasma-membrane-associated GFP–Glut4. In these experiments, the amount of GFP–Glut at the plasma membrane is expressed as a percentage of the total cellular GFP–Glut, calculated as described in the Materials and methods section. The increase in plasma-membrane-associated GFP–Glut1 was not statistically significant. For both GFP–Glut4 and Glut4–GFP, in the absence of insulin, only ~ 10% of the total cellular GFP signal was localized to the plasma membrane. In contrast, for GFP–Glut1, ~ 40% of the total cellular GFP signal was plasma-membrane localized in the basal state.

Figure 2 Co-localization of GFP-Glut4 with Texas Red transferrin or Lysotracker-Red®

After a 2 h incubation in serum-free DMEM, 3T3-L1 adipocytes expressing GFP-Glut4 were further incubated in serum-free DMEM containing either Texas Red transferrin (20 µg/ml) or Lysotracker-Red® (50 nM) for 30 min at 37 °C in a CO₂ incubator. Thereafter, cells were gently washed in KRP/glucose, transferred to the microscope stage heated to 37 °C, and images were collected using confocal microscopy. Left panel: GFP-Glut4 signal (excitation 488 nm laser, filter 505–520 nm band pass); middle panel: corresponding signal from the Texas Red transferrin or Lysotracker-Red® (excitation 545 nm laser, long-pass 590 nm filter); right panel: merged image. The results shown are representative of at least five experiments.

This pattern of intracellular localization and insulin-dependent redistribution is specific for GFP-tagged Glut4. A chimaeric GFP-Glut1 species was found to distribute between the plasma membrane and intracellular membranes in the absence of insulin, a distribution well characterized for this isoform, and did not exhibit extensive translocation to the plasma membrane (Figure 1), consistent with studies of the endogenous isoforms in 3T3-L1 adipocytes [38]. Quantification of the extent of insulin-stimulated translocation of GFP-Glut4, Glut4-GFP and GFP-Glut1 is presented in Figure 1 (lower panel). In our hands, GFP-Glut4 and Glut4-GFP behaved identically. Therefore in the following, data from experiments in which GFP-Glut4 was used are shown, but all experiments were replicated using Glut4-GFP with identical results. Collectively, these data suggest that the trafficking signals dictating the specific sorting of Glut4 and Glut1 are recognized in the context of these chimaeras.

In an effort to glean further insight into the relationship of the Glut4 storage compartment(s) with other membrane compartments, a series of fluorescent dyes were used to identify other subcellular compartments in GFP-Glut4-expressing cells. Specifically, Texas-Red-labelled transferrin was used to identify the Tfr itinerary and Lysotracker-Red® was used to identify acidic compartments (late endosomes/lysosomes). As shown in Figure 2, these compounds were effectively internalized in cells expressing GFP-Glut4. We, and others, have suggested that a significant proportion of intracellular Glut4 resides in a compartment spatially distinct from Tfr in both adipocytes and

Figure 3 Distinct localization exhibited by FAG-GFP and LAG-GFP

3T3-L1 adipocytes were microinjected with plasmid DNA encoding either FAG-GFP or LAG-GFP and incubated for 24 h before analysis. Shown are cells expressing FAG-GFP or LAG-GFP and are representative of > 10 experiments.

muscle [1,5,15,30,39]. The only partial co-localization of GFP-Glut4 or Glut4-GFP with Texas-Red transferrin (Figure 2) provides support for these observations and further suggests that the localization of the GFP-Glut4 chimaeras studied in the present work is accurately reflecting the localization of the

Figure 4 Reversal of insulin-stimulated glucose transport and Glut4 translocation in 3T3-L1 adipocytes by low-pH washing or wortmannin treatment

3T3-L1 adipocytes were incubated in serum-free DMEM for 2 h, and insulin (100 nM) was added for a further 30 min. After this time, cells were transferred to a hotplate at 37 °C and rapidly washed in KRM buffer, pH 6, for the times shown then assayed for deoxyglucose (deGlc) uptake **(A)**. **(B)** Effect of addition of 50 nM wortmannin (wort) for the times indicated, on basal and insulin-stimulated deoxyglucose (deGlc) transport. In **(A)** and **(B)** the results are the means $\pm$ S.D. of triplicate determinations. **(C)** Plasma membrane lawn assays for Glut4 performed under similar conditions. **(D)** Quantification of the results shown in **(C)**; means for three similar experiments.

endogenous protein. The results shown in Figure 2 extend further our understanding of Glut4 localization in that there was no significant overlap between the GFP–Glut4 construct and LysoTracker-Red®, strongly implying that Glut4 was not significantly localized within the late endosomal/pre-lysosomal network.

We also studied a mutant Glut4 species using this approach (Figure 3) [31–33]. FAG–GFP was observed to localize predominantly to the plasma membrane, in agreement with studies with epitope-tagged FAG stably expressed in these cells [32].

LAG–GFP expression was observed both intracellularly and, in part, at the plasma membrane (Figure 3), which was in good agreement with a range of studies examining the subcellular distribution of similar Glut4 mutants [32,33,40]. Collectively, these results strongly imply that the intracellular localization of GFP–Glut4 faithfully replicates the distribution of the endogenous protein, and that the targeting signals within Glut4 are similarly recognized within the context of the GFP–Glut4 species.

Using this system, we have demonstrated insulin-dependent translocation of GFP–Glut4 to the plasma membrane when cells

Figure 5 GFP–Glut4 is not internalized after insulin withdrawal

3T3-L1 adipocytes were microinjected with DNA encoding GFP–Glut4 and incubated for 24 h. The cells were then incubated in serum-free DMEM for 2 h, and insulin (100 nM) was added for a further 30 min. After this time, cells were either transferred to a hotplate at 37 °C and rapidly washed in KRP buffer, pH 6, for 0, 30 or 60 min or transferred to a hotplate and washed into KRP buffer, with or without 50 nM wortmannin, and incubated for 60 min before image analysis. The results are representative of three low-pH wash or four wortmannin experiments; in each experiment > 10 injected cells were examined. Quantification of the fluorescence signal in defined areas of plasma membrane as a fraction of the total cellular fluorescence from several experiments revealed that there was no significant loss of GFP–Glut4 fluorescence signal during the internalization experiments (i.e. the plasma membrane/total cellular fluorescence signal was unaffected by the experimental manipulations) (results not shown).

were treated with insulin in an incubator before image analysis (Figure 1). We therefore set out to examine the ability of GFP–Glut4 and Glut4–GFP to re-internalize from the plasma membrane after withdrawal of the insulin-stimulus (either by low pH washes, anti-insulin antibodies, or both) or in response to addition of inhibitors of PI3K, under similar conditions. In a previous study [28], we showed that a chimera similar to GFP–Glut4 did not re-internalize from the plasma membrane after removal of the insulin stimulus, but that Glut4–GFP did. However, these data were collected using CHO cells, which are not a truly insulin-responsive cell line, and, moreover, the kinetics of internalization observed did not parallel those reported for endogenous Glut4. Therefore we decided to revisit these observations, this time in a more appropriate cellular context.

In order to remove the insulin signal, we employed two distinct approaches: either washing in low pH buffer or the use of anti-insulin antibodies, or both [10,20,28]. These methods were shown to reverse insulin-stimulated glucose transport and Glut4 translocation in wild-type 3T3-L1 adipocytes with the expected half-times (Figure 4). Identical conditions were employed for cells expressing GFP–Glut4. Strikingly, no internalization of either GFP–Glut4 or Glut4–GFP was observed using any of these approaches alone or in combination (Figure 5); quantification of the level of GFP–Glut4 at the plasma membrane of these cells after these treatments did not reveal any significant reduction in signal when expressed as a percentage of the total cellular GFP–Glut4 level (results not shown). Inhibition of PI3K using wortmannin or LY294002 also functions to reverse insulin-stimulated glucose transport and Glut4 translocation (see Figure 4). However, incubation of cells with up to 250 nM wortmannin for up to 3 h did not result in appreciable internalization of GFP-tagged Glut4 from the plasma membrane (Figure 5). Quanti-

fication of these data by measurement of GFP–Glut4 fluorescence at the plasma membrane as a percentage of the total cellular GFP–Glut4 fluorescence showed no further significant internalization of GFP–Glut4 under any of the conditions employed (results not shown). These data strongly suggest that the internalization of Glut4 from the cell surface after withdrawal of the insulin-stimulus (or by inhibition of PI3K) was perturbed by the presence of GFP in the cytosolic domain of Glut4, either at the N- or C-terminus.

In order to examine the ability of GFP–Glut4-expressing cells to internalize other proteins from the cell surface, we examined the internalization of Texas-Red transferrin and Lysotracker-Red® into cells expressing GFP–Glut4 in the presence of insulin. These ligands were effectively internalized by cells in the presence of insulin (results not shown), suggesting that these cells express a functional recycling pathway capable of internalizing proteins from the plasma membrane to intracellular compartments in the presence of insulin.

We next sought to determine whether GFP-tagged Glut4 species were, like endogenous Glut4, undergoing recycling between the plasma membrane and intracellular compartments. The small numbers of injected cells used precluded direct biochemical analysis, we therefore adopted a more indirect approach. Studies have shown that depletion of K⁺ results in the accumulation of Glut4 at the plasma membrane, probably as a consequence of interruption of the recycling pathway [41]. We incubated cells expressing GFP–Glut4 in K⁺-depleted or control buffer, and examined the levels of GFP–Glut4 associated with the plasma membrane under these conditions (Figure 6, upper panel). As illustrated, cells incubated in K⁺-depleted media exhibited consistently increased levels of GFP–Glut4 at the plasma membrane compared with control cells. Quantification of

Figure 6 K⁺-depletion resulted in the accumulation of GFP–Glut4 at the plasma membrane

3T3-L1 adipocytes expressing GFP–Glut4 were washed into KRP buffer with or without K⁺. After 2 h incubation on a hotplate, cells were imaged in the same buffer by confocal microscopy using the 488 nm laser for excitation of the GFP. (Upper panel) Representative cells from control and K⁺-depleted conditions. In the cells incubated in K⁺-depleted buffer, increased GFP–Glut4 at the plasma membrane was observed in 92% of the cells examined (*n* = 76 cells in three experiments). (Middle panel) Quantification of GFP–Glut4 at the plasma membrane after either 1 or 2 h incubation in K⁺-depleted buffer, expressed as a fold increase over the levels of GFP–Glut4 at the plasma membrane in control cells. For comparison, the level of GFP–Glut4 at the plasma membrane in insulin-stimulated cells is also shown. * indicates a significant increase over Control cells (*P* = 0.005), and ***P* < 0.05. (Lower panel) Accumulation of endogenous Glut4 at the cell surface in 3T3-L1 adipocytes treated as described above for GFP–Glut4-injected cells (the results are from a representative experiment; similar results were obtained in further experiments).

Figure 6 For legend, see opposite.

the accumulation of GFP–Glut4 at the plasma membrane in these cells is shown in Figure 6 (middle panel); a statistically significant increase was observed at 2 h of incubation in K⁺-depleted buffer. These results are suggestive of recycling of GFP–Glut4 in the absence of insulin. Using the same experimental conditions, we examined the distribution of endogenous Glut4 in 3T3-L1 adipocytes by whole-cell immunofluorescence (Figure 6, lower panel). As shown, K⁺ depletion resulted in the accumulation of endogenous Glut4 at the cell surface, validating the concept that the morphological assay of GFP–Glut4 accumulation at the cell surface is reflecting the behaviour of the endogenous protein. Although we interpret these data to imply that low potassium levels are disrupting endosomal recycling, it is possible that these effects arise as a consequence of cellular stress induced by changes in membrane potential, sodium gradients and the activity of membrane transporters that utilize the sodium gradient. However, studies with primary rat adipocytes [41] have shown that the effect of potassium is to modulate recycling of Glut4 through the endosomal system, hence we argue that the responses of GFP–Glut4 to potassium depletion observed in the present work reflect the ability of this protein to recycle in the absence of insulin.

It has been shown that Glut4 undergoes constitutive recycling between the plasma membrane and intracellular compartments in the basal state [7,8,10]. The partial overlap between GFP–Glut4 and TfR-positive intracellular membranes (Figure 2) and the observation that K⁺-depletion results in the accumulation of GFP–Glut4 at the cell surface (Figure 6) support the notion that GFP–Glut4 is undergoing constitutive recycling between the plasma membrane and intracellular membranes in the absence of insulin. If the mechanism of GFP–Glut4 internalization in the basal state were the same as that employed to internalize GFP–Glut4 after withdrawal of the insulin signal, then the results shown in Figure 5 would imply that GFP–Glut4 should accumulate at the plasma membrane in the basal state. The data of Figure 1 (obtained > 24 h after microinjection) show that this is not observed. Hence, in the absence of insulin, GFP–Glut4 appears to traffic similarly to the endogenous protein and, also like the endogenous protein, exhibits insulin-stimulated translocation to the plasma membrane (Figure 1). In contrast, after insulin withdrawal or inhibition of PI3K, GFP–Glut4 does not exhibit appreciable internalization up to 6 h after removal of the insulin stimulus, or up to 3 h after addition of wortmannin (Figure 5). These observations are not a function of laser toxicity, as the insulin withdrawal or wortmannin additions were made to cells either in a CO₂ incubator or on a 37 °C hot-plate, and the cells were not exposed to laser illumination until the times indicated in the Figures. Nor is this phenomenon restricted to GFP appended to the N-terminus of Glut4, as similar phenomena were seen with Glut4–GFP (results not shown). It has also been suggested that GFP expression may be toxic to certain cells [42]. The fact that, in all other respects, GFP–Glut4 traffics apparently normally and responds well to insulin would appear to mitigate against this being an issue in this system. Rather, we argue that these observations imply distinct mechanisms of GFP–Glut4 internalization in the basal state compared with post-insulin withdrawal.

Such a conclusion presupposes that GFP–Glut4/Glut4–GFP are faithfully mimicking the trafficking of the endogenous Glut4 protein. Clearly, we cannot definitively demonstrate this, as we are limited by the very small numbers of cells expressing the protein after microinjection. However, we suggest that the following data support the notion that GFP–Glut4 is a reasonable model for endogenous Glut4 trafficking. (i) GFP–Glut4 exhibits significant insulin-stimulated translocation to the plasma mem-

brane. Others have argued that newly synthesized Glut4 traffics from the TGN to the plasma membrane and is then sorted into an insulin-responsive compartment [43]. The fact that GFP-tagged Glut4 is capable of marked insulin-stimulated translocation strongly implies that this trafficking is operating correctly for the GFP-tagged species. (ii) Glut1–GFP, in contrast, is differentially distributed between the plasma membrane and intracellular membranes. (iii) Mutations in the Glut4 primary sequence perturb targeting in a fashion similar to that reported for epitope-tagged Glut4. (iv) GFP–Glut4 exhibits partial overlap with the TfR and almost no overlap with lysosomal markers. (v) K⁺-depletion results in the accumulation of GFP–Glut4 at the plasma membrane, suggesting that GFP–Glut4 is recycling in the absence of insulin. Furthermore, GFP–Glut4 exhibits extensive overlap with the aminopeptidase, vp165/IRAP [44,45], which presents a strong case for a close relationship between the trafficking of GFP–Glut4 and endogenous Glut4.

We believe that the mechanism of GFP–Glut4 internalization in the basal state is distinct from that employed after insulin withdrawal, as no GFP–Glut4 or Glut4–GFP was observed at the plasma membrane in the basal state. Rather, we suggest that our data are consistent with a mechanistic distinction between GFP–Glut4 internalization from the plasma membrane in the basal state and GFP–Glut4 internalization after insulin withdrawal. We suggest that such mechanistic distinctions may also be operating for the endogenous protein, implying that distinct mechanisms for Glut4 internalization may exist in peripheral tissues. In further support of this hypothesis, we note that the IC₅₀ for wortmannin inhibition of basal and insulin-stimulated deoxy-Glc transport are distinct, and also that the inhibition of insulin-stimulated glucose transport, observed in response to wortmannin, does not fall appreciably below the basal rate [18,37,46] (see also Figure 4). We argue that studies using GFP-tagged Glut4 can reveal hitherto unsuspected subtleties of Glut4 trafficking which may be of relevance to endogenous Glut4 cell biology.

This work was supported by grants from the Wellcome Trust, the British Diabetic Association and Tenovus (Scotland) to G.W.G. and the Medical Research Council (to J.M.T.). J.M.T. is a British Diabetic Association Research Fellow and G.W.G. is a Lister Institute of Preventive Medicine Research Fellow. L.C.C. thanks the BBSRC and Pfizer Central Research (Groton, CT, U.S.A.) for financial support for his PhD studentship.

REFERENCES

- 1 Rea, S. and James, D. E. (1997) *Diabetes* **46**, 1667–1677
- 2 Zorzano, A., Munoz, P., Camps, M., Mora, C., Testar, X. and Palacin, M. (1996) *Diabetes* **45**, S70–S81
- 3 Ploug, T., van Deurs, B., Ai, H., Cushman, S. W. and Ralston, E. (1998) *J. Cell Biol.* **142**, 1429–1446
- 4 Slot, J. W., Geuze, H. J., Gigengack, S., James, D. E. and Lienhard, G. E. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7815–7819
- 5 Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991) *J. Cell Biol.* **113**, 123–135
- 6 Slot, J. W., Garruti, G., Martin, S., Oorschot, V., Posthuma, G., Kraegen, E. W., Laybutt, R., Thibault, G. and James, D. E. (1997) *J. Cell Biol.* **137**, 1243–1254
- 7 Yang, J. and Holman, G. D. (1993) *J. Biol. Chem.* **268**, 4600–4603
- 8 Yang, J., Clark, A. E., Harrison, R., Kozka, I. J. and Holman, G. D. (1992) *Biochem. J.* **281**, 809–817
- 9 Satoh, S., Nishimura, H., Clark, A. E., Kozka, I. J., Quon, M. J., Cushman, S. W. and Holman, G. D. (1993) *J. Biol. Chem.* **268**, 17820–17829
- 10 Holman, G. D., Leggio, L. L. and Cushman, S. W. (1994) *J. Biol. Chem.* **269**, 17516–17524
- 11 Holman, G. D., Kozka, I. J., Clark, A. E., Flower, A. F., Saltis, J., Habberfield, A. D., Simpson, I. A. and Cushman, S. W. (1990) *J. Biol. Chem.* **265**, 18172–18179
- 12 Chakrabarti, R., Buxton, J., Joly, M. and Corvera, S. (1994) *J. Biol. Chem.* **269**, 7926–7933

- 13 Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992) *J. Cell. Biol.* **117**, 1181–1196
- 14 Volchuk, A., Narine, S., Foster, L. J., Grabs, D., De Camilli, P. and Klip, A. (1998) *J. Biol. Chem.* **273**, 8169–8176
- 15 Livingstone, C., James, D. E., Rice, J. E., Hanpeter, D. and Gould, G. W. (1996) *Biochem. J.* **315**, 487–495
- 16 Kandror, K. V. and Pilch, P. F. (1996) *J. Biol. Chem.* **271**, 21703–21708
- 17 Kandror, K. V., Coderre, L., Pushkin, A. V. and Pilch, P. F. (1995) *Biochem. J.* **307**, 383–390
- 18 Okada, T., Kawano, Y., Sakakibara, T., Kazeki, O. and Ui, M. (1994) *J. Biol. Chem.* **269**, 3568–3573
- 19 Yang, J., Clarke, J. F., Ester, C., Young, P. W., Kasuga, M. and Holman, G. D. (1996) *Biochem. J.* **313**, 125–131
- 20 Gibbs, E. M., Lienhard, G. E. and Gould, G. W. (1988) *Biochemistry* **27**, 6681–6685
- 21 Gerdes, H.-H. and Kaether, C. (1996) *FEBS Lett.* **389**, 44–47
- 22 Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergas, F. G. and Cormier, M. J. (1992) *Gene* **111**, 229–233
- 23 Girotti, M. and Banting, G. (1996) *J. Cell. Science* **109**, 2915–2926
- 24 Drmot, T., Gould, G. W. and Milligan, G. (1998) *J. Biol. Chem.* **273**, 24000–24008
- 25 Tarasova, N. I., Stauber, R. H., Choi, J. K., Hudson, E. A., Czerwinski, G., Miller, J. L., Pavlak, G. N., Michejda, C. J. and Wank, S. A. (1997) *J. Biol. Chem.* **272**, 14817–14824
- 26 Kaether, C. and Gerdes, H.-H. (1995) *FEBS Lett.* **369**, 267–271
- 27 Oatey, P. B., Van weering, D. H. J., Dobson, S. P., Gould, G. W. and Tavaré, J. M. (1997) *Biochem. J.* **327**, 637–642
- 28 Dobson, S., Livingstone, C., Gould, G. W. and Tavaré, J. M. (1996) *FEBS Lett.* **393**, 179–184
- 29 Martin, S., Reaves, B., Banting, G. and Gould, G. W. (1994) *Biochem. J.* **300**, 743–749
- 30 Martin, S., Tellam, J., Livingstone, C., Slot, J. W., Gould, G. W. and James, D. E. (1996) *J. Cell Biol.* **134**, 625–635
- 31 Melvin, D. R., Marsh, B. J., Martin, S., Walmsley, A. R., James, D. E. and Gould, G. W. (1998) *Biochemistry* **38**, 1456–1462
- 32 Marsh, B. J., Alm, R. A., McIntosh, S. R. and James, D. E. (1995) *J. Cell Biol.* **130**, 1081–1091
- 33 Marsh, B. J., Martin, S., Melvin, D. R., Alm, R. A., Martin, L. J., Gould, G. W. and James, D. E. (1998) *Am. J. Physiol.* **275**, E412–E422
- 34 Martin, L., Shewan, A., Millar, C. A., Gould, G. W. and James, D. E. (1998) *J. Biol. Chem.* **273**, 1444–1452
- 35 Holman, G. D. and Kasuga, M. (1997) *Diabetologia* **40**, 991–1003
- 36 Clark, A. E., Holman, G. D. and Kozka, I. J. (1991) *Biochem. J.* **278**, 235–241
- 37 Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M. and Holman, G. D. (1994) *Biochem. J.* **300**, 631–635
- 38 Calderhead, D. M., Kitagawa, K., Tanner, L. I., Holman, G. D. and Lienhard, G. E. (1990) *J. Biol. Chem.* **265**, 13800–13808
- 39 Ralston, E. and Ploug, T. (1996) *J. Cell. Sci.* **109**, 2967–2978
- 40 Verhey, K. J., Yeh, J. I. and Birnbaum, M. J. (1995) *J. Cell Biol.* **130**, 1071–1079
- 41 Nishimura, H., Zarnowski, M. J. and Simpson, I. A. (1993) *J. Biol. Chem.* **268**, 19246–19253
- 42 Liu, H.-S., Jan, M.-S., Chou, C.-K., Chen, P.-H. and Ke, N.-J. (1999) *Biochem. Biophys. Res. Commun.* **260**, 712–717
- 43 Al-Hasani, H., Hinck, C. S. and Cushman, S. W. (1998) *J. Biol. Chem.* **273**, 17504–17510
- 44 Kandror, K. V., Yu, L. and Pilch, P. F. (1994) *J. Biol. Chem.* **269**, 30777–30780
- 45 Martin, S., Rice, J. E., Gould, G. W., Keller, S., Slot, J. W. and James, D. E. (1997) *J. Cell. Sci.* **110**, 2281–2291
- 46 Jess, T. J., Belham, C. M., Thomson, F. J., Scott, P. H., Plevin, R. J. and Gould, G. W. (1996) *Cell Signalling* **8**, 297–304

Received 30 June 1999/1 September 1999; accepted 21 September 1999