Subcellular trafficking kinetics of GLUT4 mutated at the N- and C-termini

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The glucose transporter isoform, GLUT4, has been expressed in Chinese hamster ovary clones and its subcellular trafficking has been determined following labelling at the cell surface with the impermeant bis-mannose photolabel, 2-*N*-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA). ATB-BMPA-tagged GLUT4 leaves the cell surface rapidly and equilibrates to give an internal/surface distribution ratio of approx. 3.5 after 60 min. GLUT4 in which the N-terminal phenylalanine-5 and glutamine-6 are mutated to alanine N-(FQ-AA) and in which the C-terminal leucine-489 and -490 are mutated to alanine C-(LL-AA) have low internal/surface ratios of 0.64 and 1.24 respectively. If all cell-surface transporters are able to recycle, as would be the case for a two-pool recycling model with a single intracellular pool, then analysis suggests that the wild-type GLUT4 distribution ratio is dependent on endocytosis and exocytosis rate constants of 0.074 and 0.023 min⁻¹. These values are similar, but not identical, to those found for GLUT4 trafficking in adipocytes. The distribution of the N-(FQ-AA) transporter appears to be due to a decrease in endocytosis with reduced intracellular retention, while the distribution of the

C-(LL-AA) transporter appears to be mainly due to poor intracellular retention. These results are also considered in terms of a consecutive intracellular pool model in which GLUT4 targeting domains alter the distribution between recycling endosomes and a slowly recycling compartment. In this case the more rapid apparent exocytosis of the mutated GLUT4s is due to their failure to reach a slowly recycling compartment with a consequent return to the plasma membrane by default. It is suggested that overexpression of transporters increases the proportion that are recycled in this way. Wortmannin is shown to decrease glucose transport activity and cell-surface photolabelled transporters in a manner consistent with an inhibition of transporter recycling. Studies on the rate of loss of transport activity and ATB-BMPA-tagged transporter in wortmannin-treated cells confirm that the N-(FQ-AA) mutant is endocytosed more slowly than the wild-type GLUT4. Taken together, these results suggest that mutation at either the N- or the C-terminal domain can reduce movement to a slowly recycling intracellular compartment but that neither domain alone is entirely sufficient to produce wild-type GLUT4 trafficking behaviour.

INTRODUCTION

In 1980, Suzuki and Kono [1] and Cushman and Wardzala [2] reported that glucose transporter isoforms (GLUTs) in non-stimulated rat adipose cells are located in a large intracellular pool and can be recruited to the plasma membrane in response to insulin. This important feature of glucose transporter translocation has now been shown to be mainly associated with the distinct properties of the GLUT4 isoform which, in non-stimulated adipose cells, has a greater propensity to remain localized in intracellular vesicles than the GLUT1 isoform also present in adipose cells [3–6]. Consequently, insulin stimulates the translocation of both glucose transporter isoforms, the effect on GLUT4 is significantly greater than on GLUT1 (≈ 20 - and 4-fold above the non-stimulated levels respectively).

The greater intracellular sequestration of GLUT4 has also been shown in heterologous expression systems including oocytes [7–9], COS cells [10,11], Chinese hamster ovary (CHO) cells [11,12], 3T3 fibroblasts and PC12 cells [13]. The implication from these studies is that GLUT4 has a unique amino acid sequence or sequences within its primary structure which direct its targeting to a specialized intracellular location. Studies on the trafficking of membrane proteins and receptors have led to the recognition of three distinct types of motif that can direct intracellular trafficking. These are a β -turn with an aromatic residue at the turn [14,15], a di-leucine motif [16] and, as in the

case of TGN38, both a transmembrane domain and a β -turn type of motif [17,18]. GLUT4 has several potential targeting domains and evidence has been presented suggesting that all three types of motif may be involved in GLUT4 trafficking. Piper et al. [19] have shown that GLUT1/GLUT4 and asialoglycoprotein receptor/GLUT4 chimera with the GLUT4 N-terminal region have increased distribution to the cell interior. The motif in the GLUT4 N-terminal region is of the β -turn type with an aromatic phenylalanine residue at position 5. Piper et al. [20] showed that substitution of this phenylalanine reduced the internalization. Asano et al. [12] have suggested that central sections of GLUT4 in the region of transmembrane helices 7 and 8 are important for targeting while Verhey and co-workers [13,21], Corvera et al. [22] and Haney et al. [23] have presented data from studies of the GLUT1/GLUT4 chimera that suggest that the C-terminus is important. These latter groups suggest that the C-terminus is the most important motif, see no evidence for an involvement of the N-terminus in their expression systems, and suggest that the di-leucines within this GLUT4 C-terminal section are the essential targeting residues. More recently, Verhey et al. [24] and Marsh et al. [25] have shown that both N- and C-terminal motifs may be involved in targeting in insulin-responsive 3T3-L1 cells.

Most previous studies of GLUT4 targeting motifs have measured the ratio of distribution between the cell surface and the intracellular membranes and have not in general studied the

Abbreviations used: ATB-BMPA, 2-*N*-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(*D*-mannos-4-yloxy)-2-propylamine; GLUT, glucose transporter isoform; GT4TR, GLUT4/transferrin receptor; CHO, Chinese hamster ovary; I/S, internal/surface ratio; KRHB, Krebs–Ringer Hepes buffer; TGN, *trans*-Golgi network.

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kinetics of equilibration of transporter between these locations. In the study described here we have directly followed the trafficking kinetics of GLUT4 selectively mutated at residues within the putative N- and C-terminal targeting domains. The selective mutation of these residues was considered a complementary approach to the studies on GLUT4 chimera cited above. The selectively mutated GLUT4 transporters have been tracer-tagged by the impermeant photolabel, 2-*N*-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(*D*-mannos-4-yloxy)-2-propylamine (ATB-BMPA), and the kinetics of internalization have been followed. The trafficking kinetics have been analysed in terms of models in which there is either a single intracellular pool, or in which there are two consecutive intracellular pools [26]. It is suggested that the latter model can account more readily for much of the anomalous trafficking behaviour of GLUT4 and its mutants both in insulin-responsive cells and also in the CHO cells described here.

We have previously shown [27] that wortmannin can inhibit the constitutive recycling, as well as the insulin-regulated recycling, of glucose transporters in 3T3-L1 cells. We describe here how this effect occurs in poorly insulin-responsive CHO cells and how this reagent can be used as a tool for resolving aspects of the transporter translocation kinetics.

EXPERIMENTAL

Materials

Wortmannin was from Sigma and was stored in DMSO. 2-Deoxy-[2,6-³H]D-glucose was from Amersham International. ATB-[2-³H]BMPA (specific radioactivity 10 Ci/mmol) was synthesized as described [28].

Site-directed mutagenesis of human GLUT4 cDNA and expression in CHO cells

A full-length human GLUT4 cDNA was kindly provided by Dr. G. I. Bell (University of Chicago, Chicago, IL, U.S.A.). Point mutations were introduced following the method of Kunkel [29]. The template for mutagenesis was prepared in *Escherichia coli* RZ1032 and mutagenesis was carried out by using the mutagenic primers, (5'-GGAGCCTATCTGTGCGGCGCCGACGG-3') for the N-terminal mutant with Phe-5 and Gln-6 replaced by alanine, N-(FQ-AA), and (5'-CACCTCCTGCTCTGCAGCAGAGGGTGTC-3') for a C-terminal mutant with Leu-489 and Leu-490 replaced by alanine, C-(LL-AA). Suitable mutant clones were selected and their sequences were confirmed by dideoxynucleotide sequencing in M13. The mutated GLUT4 cDNA was subcloned into a *Hind*III/*Xba*I site of pRC/CMV which has the human cytomegalovirus IE1 promoter and the bacterial neomycin resistance gene fused to the TV40 promoter. The wild-type and mutant GLUT4 cDNAs were transfected by the calcium phosphate method into CHO-K1 cell lines which were maintained in Ham's F-12 medium containing 10% (v/v) fetal calf serum. The clones which obtained neomycin resistance were selected with 500 µg/ml of the neomycin derivative G418 (Gibco). The cells were subsequently selected by application of Western blotting to identify clones which express these glucose transporters. This was carried out as previously described [30] except that the antibody used was raised against a peptide in a region within the central intracellular loop of human GLUT4. This peptide, CKRLTGWADVSGVLAELKDE, corresponds in sequence to residues 245–263. Mutant clones expressing similar amounts of protein to the wild-type clone were selected for further study.

Glucose transport activity

Cells were grown to confluence in 35-mm-diam. culture dishes for 2–3 days and then washed three times in Krebs–Ringer Hepes buffer (KRHB), containing 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄ and 10 mM Hepes, pH 7.4. 2-Deoxy-[2,6-³H]D-glucose was then added to give a final assay concentration of 0.05 mM in 1 ml of KRHB and incubated at 37 °C. Uptake was terminated at 2 min by an addition of 1 ml/well of ice-cold PBS containing 0.3 mM phloretin, followed by three rapid washes in ice-cold PBS/phloretin. Following the arrest of transport, cells were solubilized with 1 ml of 0.1 M NaOH and the extract was added to scintillant for estimation of radioactivity. Zero-time uptake was determined by adding phloretin before the transported substrate.

ATB-BMPA photolabelling

Confluent cells in 60-mm-diam. dishes were washed three times in KRHB and labelled at 18 °C with 500 µCi of ATB-[2-³H]BMPA in 0.5 ml of KRHB. Samples were covered by a glass plate and irradiated for 1 min using 350 nm light from a Rayonet RPR-100 photochemical reactor. Following irradiation the dishes were rapidly washed four times and then maintained in KRHB containing 2 mM D-glucose at 37 °C for the times indicated in the Figure legends. Cells were then homogenized in 2 ml of TES buffer (10 mM Tris/HCl, 0.5 mM EDTA, 255 mM sucrose, pH 7.2) with 15 full strokes using a tight-fitting Potter–Elvehjem homogenizer (Thomas Scientific). Since ATB-BMPA is a cell-impermeant photolabel, the cell-surface labelling obtained at 18 °C was used as a plasma membrane marker to optimize the homogenization and differential centrifugation procedures. The procedure described produces good recovery of plasma membrane while minimizing the cross-over to the low-density microsome fraction. Crude plasma membranes were sedimented at 16000 *g*_{max} for 20 min, rehomogenized in 0.3 ml of TES and then separated on a 1.12 M sucrose cushion in 0.6 ml of TES at 66300 *g*_{max} for 20 min. The purified plasma membrane was then sedimented at 86000 *g*_{max} for 9 min. The first supernatant was recentrifuged at 20000 *g*_{max} for 20 min, this pellet was discarded, and then at 541000 *g*_{max} for 17 min to isolate the intracellular membranes. Membrane samples were solubilized in an electrophoresis sample buffer containing 10% SDS, 6 M urea and 10% mercaptoethanol and were subjected to electrophoresis on 9% acrylamide gels using a discontinuous buffer system [31]. Radioactivity in gel slices was extracted and quantified as described [32].

Analysis of trafficking kinetics

The data on the loss of ATB-BMPA-tagged GLUT4 from the cell surface under steady-state conditions have been subjected to two types of analysis. The first assumes that there are only two pools of glucose transporters, one in the plasma membrane and the other in the intracellular membrane system of recycling endosomes (Figure 1A). In this case the reduction in tracer-tagged plasma membrane transporters (TP) is described by eqn. (1):

$$TP = (k_{ex} + k_{en} \cdot \exp^{-(k_{en} + k_{ex}) \cdot t}) / (k_{ex} + k_{en}) \quad (1)$$

Second, we have determined whether the data can also be described by the consecutive intracellular pool model [26] in which recycling can occur either from the sorting endosome (TE) or the second tubulovesicular compartment (TV) (Figure 1B).

Figure 1 Analysis of glucose transporter recycling kinetics

In (A) there is a single intracellular pool, TE (= Te on Figure). Translocations into and out of this pool are described by the rate constants *ken* and *kex*, respectively. In (B) there are two consecutive intracellular pools. Translocations into and out of the first, TE, compartment are described by the rate constants *ken* and *krc*, respectively, while trafficking into and out of the second, TV (= Tv on Figure), compartment is described by the rate constants *ksq* and *kex*, respectively.

The differential equations describing this model are:

$$TP' = kex \cdot TV + krc \cdot TE - ken \cdot TP \quad (2.1)$$

$$TE' = ken \cdot TP - (krc + ksq) \cdot TE \quad (2.2)$$

$$TV' = ksq \cdot TE - kex \cdot TV \quad (2.3)$$

where TP, TE and TV are the proportion of transporters at the cell surface, the sorting endosome compartment or the sequestered vesicle compartment and the rate constants *ken*, *krc*, *ksq* and *kex* represent the endocytosis, the recycling, the sequestration and exocytosis rate constants respectively. These equations are most easily integrated numerically and we have used the 'Scientist' program from MicroMath, Salt Lake City, UT, U.S.A. which allows curve-fitting to sets of differential equations. Because the data were not sufficiently precise to allow unconstrained least-squares fitting to all four parameters of the model, the endocytosis rate constant from the two-pool model was used as a fixed parameter and the remaining three parameters were then calculated by least-squares fitting to eqns. (2). This approach does not provide unbiased estimates of all rate constants but instead provides a fit to the data showing that the model can simulate the data.

The effect of wortmannin on the net loss of transporters from the plasma membrane can be described by the equation:

$$TP = [kex \cdot (1 - \exp^{-(kex+ken) \cdot t})] / (kex + ken) + TP_0 \cdot \exp^{-(kex+ken) \cdot t} \quad (3)$$

where TP_0 is the initial level of transporter in the plasma membrane [26]. The TP_0 value was assumed to be the same as the level of isotope distribution of ATB-BMPA-tagged transporters at steady state.

Although model-dependent curve-fitting was carried out the equations used have the same form as the model-independent eqn. (4).

$$TP = (1 - R) \exp^{-k \cdot t} + R \quad (4)$$

where *k* is the rate constant related to the half-time of the change in surface activity and *R* is the equilibration level of activity. Note that *k* is $(kex + ken)$ and *R* is $kex / (kex + ken)$ and for the two-pool model.

Figure 2 Expression of GLUT4 mutants in CHO cells

Total cellular membranes were isolated from non-transfected cells (lane 1), wild-type GLUT4 (lane 2) and N-(YQ-AA) (lane 3) and C-(LL-AA) (lane 4) GLUT4 mutant CHO clones and the transporters were then detected and quantified using an antiserum raised against a peptide corresponding to a region in the central cytosolic loop.

Figure 3 Translocation of ATB-BMPA-GLUT4 between subcellular membrane fractions

GLUT4 was cell-surface photolabelled with ATB-BMPA at 18 °C and membrane fractions were either obtained immediately (●) or following incubation at 37 °C for 30 min (▲). Purified plasma membrane (A) and low-density microsomal fractions (B) were then isolated. Labelled GLUT4 was then resolved by electrophoresis on 9% polyacrylamide/SDS gels.

RESULTS

Expression of transfected glucose transporters in CHO cells

GLUT1, GLUT4 and GLUT4 mutant glucose transporters were expressed in CHO cells at levels which were severalfold higher than the level of endogenous GLUT1. A typical Western blot analysis is shown in Figure 2. In these experiments the GLUT4

Figure 4 Internalization of ATB-BMPA-tagged GLUT4 mutants in CHO cells

Wild-type GLUT4 ($\blacktriangle$) and its N-(YQ-AA) ($\bullet$) and C-(LL-AA) ($\triangle$) mutants were cell-surface photolabelled with ATB-BMPA. The decrease in plasma membrane label was then used to calculate the internal/surface distribution ratio. Results are the mean $\pm$ S.E.M. from three experiments except the 45 and 60 min points which are means for two experiments. The fitted curves are from models of trafficking in which there are either two intracellular pools (wild-type, four-parameter fit) or a single pool [N-(YQ-AA) and C-(LL-AA) GLUT4 mutants, two-parameter fit].

was detected by an antibody that recognizes the central loop region. Mutants which are altered at the C-terminus could not be reliably compared when employing the more conventionally used C-terminal antibody. Levels of each transporter were calculated as a percentage of the wild-type GLUT4 level. The clones expressing GLUT4 mutated at the N-terminus N-(FQ-AA), or at the dileucine residue at the C-terminus C-(LL-AA) had expression levels which were 95.6 ± 5.1 and 134.4 ± 5.5 % (mean $\pm$ S.E.M.) of the wild-type level respectively. By comparison of GLUT4 membranes from transfected CHO cells and from endogenous GLUT4 in 3T3-L1 cells it was found that CHO cells express 2–3-fold higher levels of GLUT4. The wild-type GLUT1 had, as previously shown [30], an expression level that was 7-fold higher than the non-transfected clone.

Steady-state trafficking of transfected glucose transporters in CHO cells

The impermeant photolabel ATB-BMPA has been used to tag cell-surface GLUT4. Following irradiation, the tagged GLUT4 leaves the plasma membrane fraction and the amount of label recovered in the light microsome fraction of the cell increases (Figures 3A and 3B). A series of experiments showed that the total recovered GLUT4 from plasma membrane and low-density microsome fractions stays constant and equal to 1914 ± 182 and 1885 ± 262 d.p.m. (mean $\pm$ S.E.M. from four experiments) immediately after labelling and after 30 min equilibration respectively. These results therefore show that the fractionation procedure results in no net loss of transporter. In addition, the decrease in label from the plasma membrane can be fully accounted for by its recovery in the low-density microsome fraction.

To compare the trafficking of the GLUT4 mutants, only the plasma membrane fraction was recovered. We have plotted trafficking data as internal/surface ratio (I/S) versus time. This plot more clearly shows any tendency of the data to depart from a single exponential function than the previously used TP versus

Table 1 Effect of wortmannin on GLUT4 trafficking kinetics in transfected CHO cells

Trafficking kinetic parameters using ATB-BMPA photolabel were determined by fitting to eqn. (1). The transport data were fitted to eqn. (3) using the ATB-BMPA equilibrium label distribution for TP₀. The sum of *kex* and *ken* gives a rate constant related to the half-time of the trafficking. This rate constant, the half-time and the distribution ratio *kex*/(*kex* + *ken*) are model-independent parameters (compare eqns. 1 and 4). Parameter values are the mean and S.E.M. of the indicated number of experiments.

Clone	Protocol	<i>kex</i> (min ⁻¹)	<i>ken</i> (min ⁻¹)
GLUT4 (wild type)	ATB-BMPA labelling (<i>n</i> = 3)	0.023 ± 0.009	0.074 ± 0.010
	Labelling + wortmannin (<i>n</i> = 2)	0.016	0.082
	Transport + wortmannin (<i>n</i> = 3)	0.010 ± 0.004	0.117 ± 0.021
GLUT4 N-mutant (F5,Q6 $\rightarrow$ AA)	ATB-BMPA labelling (<i>n</i> = 3)	0.096 ± 0.018	0.061 ± 0.002
	Labelling + wortmannin (<i>n</i> = 1)	0.023	0.047
	Transport + wortmannin (<i>n</i> = 4)	0.029 ± 0.002	0.053 ± 0.006
GLUT4 C-mutant (L489,L490 $\rightarrow$ AA)	ATB-BMPA labelling (<i>n</i> = 3)	0.058 ± 0.011	0.072 ± 0.008
	Labelling + wortmannin (<i>n</i> = 1)	0.027	0.086
	Transport + wortmannin (<i>n</i> = 3)	0.028 ± 0.004	0.092 ± 0.011

time plot. The I/S values are related to the proportion of transporters in the plasma membrane (TP) as follows:

$$TP = S/(I + S) \text{ and } I/S = 1/TP - 1$$

The wild-type GLUT4 leaves the plasma membrane with a half-time of ≈ 7 min and reaches an equilibrium point where the ratio of transporters in the intracellular pools and plasma membrane pools is ≈ 3.5 after 60 min (Figure 4). This is an apparent equilibrium point as the proportion of intracellular label very slowly increases to ≈ 6.0 with incubations of 120 min (results not shown). As a first approximation, we have determined the kinetics of trafficking using a model that assumes that there is a single intracellular pool (eqn. 1). Using this analysis, the rate constants for the endocytosis and exocytosis of the wild-type GLUT4 are 0.074 and 0.023 min⁻¹ (Table 1). The former rate constant is slightly lower, while the latter is slightly higher than the GLUT4 present in 3T3-L1 cells.

Since, in the case of the wild-type GLUT4, these results were insufficiently precise to determine directly the four parameters for the consecutive pool model, we have used information obtained assuming that trafficking occurs through a single intracellular pool (Table 1) to derive approximate parameters for entry into the second compartment. We have fixed the endocytosis rate constant at 0.091 min⁻¹ (the mean value for wild-type GLUT4 from Table 1). Least-squares fitting to the data in Figure 3 and the 120 min data point, then gives the remaining parameters. The calculated *krc* = 0.047 ± 0.010 min⁻¹, *ksq* = 0.017 ± 0.011 min⁻¹ and *kex* = 0.002 ± 0.004 min⁻¹ are obtained. The S.E.M. values for the parameter estimates are large but this result is interesting because the value for *krc* is similar to values for recycling of transferrin receptors [33] and the apparent *kex* rate constants of GLUT4 mutants (Table 1). Therefore, the two-pool analysis, although inadequate to account for all aspects of glucose transporter trafficking, can provide useful clues to the numerical values of rate constants in a more complex model which would otherwise be difficult to derive.

Unlike the wild-type GLUT4, the C-(LL-AA) and N-(FQ-AA) mutants are not so extensively internalized over long time periods and reach equilibrium I/S ratios of 1.24 and 0.64 respectively. Curve-fitting to eqn. (1) (Table 1) gives the apparent rate constants for endocytosis and exocytosis. The mutation in the di-leucine at the C-terminus of GLUT4 produces little

alteration in the endocytosis rate constant ($k_{en} = 0.072 \text{ min}^{-1}$), while the apparent exocytosis is more rapid ($k_{ex} = 0.058 \text{ min}^{-1}$) than for wild-type GLUT4. However, as discussed above the difference in apparent exocytosis of the mutant may occur because it does not significantly enter the second intracellular compartment. Since the LL-mutated GLUT4 is more extensively internalized than the N-(FQ-AA) mutant, additional targeting regions other than LL are necessary for wild-type levels of distribution to the intracellular membranes.

Use of the photolabel suggests that the N-(FQ-AA) mutant has a slightly slower endocytosis rate constant than the wild type while the exocytosis rate constant is much higher (0.096 min^{-1}). The relative differences in the endocytosis rate constant between the wild-type GLUT4 and the N-mutant are comparable with those previously determined for the trafficking of a GT4TR chimera in which the N-terminus of GLUT4 is inserted [33]. The endocytosis rate constants of the wild-type and an F5-A mutant GT4TR chimera are 0.06 and 0.03 min^{-1} respectively [33]. The wild-type and the mutated GT4TR chimera are recycled rapidly with a rate constant of $\approx 0.05 \text{ min}^{-1}$ [33]. Consequently the wild-type GT4TR chimera is not as extensively sequestered as shown here for the wild-type GLUT4. It appears that additional targeting regions other than the N-terminus are necessary to produce the low apparent exocytosis rate constant for wild-type GLUT4. Therefore, neither the N- nor the C-terminal domain alone is sufficient to produce wild-type subcellular trafficking behaviour.

Effects of wortmannin on glucose transporter recycling in CHO cells

We have previously shown that in 3T3-L1 cells wortmannin can inhibit the constitutive and insulin-regulated recycling of both GLUT1 and GLUT4 [27]. To demonstrate this effect in the CHO cell system, we have studied the effects of wortmannin on the recycling of ATB-BMPA-tagged transporters (Table 1). The wortmannin treatment appears to produce an inhibition of label exocytosis for all three GLUT4 clones.

When CHO cells expressing wild-type GLUT4 or its targeting mutants are treated with wortmannin there is extensive loss of glucose transport activity (Figure 5A). Therefore, it seems that wortmannin can be used as a simple assay tool to provide relatively independent evidence for some of the conclusions arising from studying the steady-state trafficking of photolabelled transporters. However, to use the effects of wortmannin perturbation of trafficking to calculate endocytosis and exocytosis rate constants, it is necessary to know the proportion of transporters at the cell surface at the beginning of the experiment. For the calculated rate constants in Table 1, least-squares fitting has been carried out using the ATB-BMPA equilibrium data to calculate TP_0 , the starting point for curve-fitting to eqn. (3). The validity of this approach is seen in the marked similarity of the rate constants obtained from transport data with starting points (TP_0) of 0.24 , 0.45 and 0.61 for the wild-type, the C-(LL-AA) and N-(FQ-AA) mutants respectively, and from steady-state photolabelling assays with all the tagged-transporters initially in the plasma membrane ($TP_0 = 1.0$ for all three transporters). Comparing the data from both labelling and transport activity suggests that wortmannin reduces recycling of all transporters with a small increase in endocytosis of wild-type GLUT4. However, the individual values for the apparent endocytosis rate constant for transport perturbation following wortmannin treatment of wild-type GLUT4 were 0.166 , 0.103 and 0.081 min^{-1} , and therefore within the range of the mean endocytosis rate constant obtained from photolabelling data.

Figure 5 Effect of wortmannin on the glucose transport activity associated with mutant GLUT4

CHO clones were treated with $1 \mu\text{M}$ wortmannin at 37°C and the net loss of 2-deoxy-D-glucose transport activity that was attributable to in (A), cell-surface GLUT4 (●), the N-(YQ-AA) (○) and C-(LL-AA) (▲) GLUT4 mutants was determined at the indicated times. In (B), the wortmannin-induced decrease in transport activity in CHO cells transfected with wild-type GLUT1 (▲) was compared with that occurring in non-transfected CHO cells (●). The decreases in transport activity were fitted to eqn. (3).

In the presence of wortmannin, where recycling is inhibited, the role of the N-terminus in endocytosis can be more clearly resolved (Table 1). Taken together, the data from labelling and transport activity suggest that only the N-(FQ-AA) mutant has a slowed endocytosis rate constant which is 41% slower than for the wild-type.

Wortmannin treatment of CHO clones expressing wild-type GLUT1 also produces a net loss of glucose transport activity (Figure 5B). However, the net loss of activity is very slow and occurs with an apparent rate constant of $\approx 0.02 \text{ min}^{-1}$. Because the internalization is very slow it was not possible to use curve-fitting to resolve whether k_{en} or k_{ex} is altered as either can influence the net internalization (eqn. 3). The composite rate constant value for the net internalization is similar to that which has been reported for bulk membrane flow and internalization of transferrin receptors which are truncated in the targeting motif [33]. The rate constant for internalization is much slower than for endocytosis of GLUT1 in rat adipocytes and 3T3-L1 cells where the endocytosis rate constant is $\approx 0.09 \text{ min}^{-1}$ [27,34–36]. The slow net internalization of exogenous GLUT1 in CHO cells may be a consequence of its overexpression which may saturate a

trafficking step. Evidence that this may indeed be the case is shown in Figure 5(B) where the net losses of transport activity in wild-type and non-transfected CHO cells are compared. The loss of endogenous GLUT1 and associated glucose transport activity is much more rapid than for the overexpressed GLUT1. The rate constant for loss of endogenous GLUT1 in CHO cells is similar to that calculated for endocytosis of GLUT1 in 3T3-L1 cells.

DISCUSSION

Studies on the steady-state distribution of GLUT4 in heterologous expression systems [7–13] suggest that these systems may have some capacity to recognize GLUT4 and target this isoform to intracellular membranes as occurs in insulin-responsive cells. It is apparent that some aspects of trafficking such as the clathrin-dependent endocytosis step are similar in the two systems [33]. Indeed, the rate constant for endocytosis of wild-type GLUT4 obtained in the present study is similar to, but slightly slower than, that obtained for endogenous GLUT4 in rat adipocytes and in 3T3-L1 cells [27,34–36].

Analysis of data for the trafficking of ATB-BMPA-tagged N- and C-mutant GLUT4 suggests that these transporters have rates of trafficking that are similar to those reported for endosome recycling of transferrin receptors (with and without the GLUT4 domains) [33]. These comparisons suggest that the mutated GLUT4 may recycle in a manner similar to the well-characterized route for recycling of transferrin receptors and that only the wild-type GLUT4 can be sorted out of this system into a more slowly recycling compartment. The sequestration of GLUT4 into this second compartment would give an apparent slowing of exocytosis in comparison with its N- and C-terminal mutants.

To quantitatively determine whether this consecutive intracellular pool model can account for trafficking of GLUT4 and its mutants we have set up equations describing the model (Figure 1B). Simulation of the expected behaviour of this model indicates that a slow sequestration within a second compartment can slow the return of transporters to the plasma membrane because this process depletes the endosomes. Therefore, less GLUT4 is available for recycling via the endosome route although the rate constant for this step (*k_{rec}*) can be the same for all transporters. The difference between the distribution of N-(FQ-AA) and C-(LL-AA) mutants is then due only to the slower endocytosis rate constant (*k_{en}*) of the former isoform.

We have carried out a similar analysis of the trafficking of GT4TR chimera with a GLUT4 C-terminus (T. E. McGraw, S. W. Cushman and G. D. Holman, unpublished work). This chimeric protein behaves in a similar manner to that of the wild-type GLUT4 described here in that its internalization process cannot be described by recycling through a single intracellular pool. Rather, there is clear evidence that after an initial rapid internalization there is a slow rate of entry into a second compartment.

The results now showing kinetic evidence for consecutive trafficking pools in CHO cells lead to questions concerning the differences between GLUT4 behaviour in CHO and in insulin-target tissues. Simulation data using the GLUT4 rate constants obtained in the present study suggest that the distribution of tagged GLUT4 between the TE and TV pools may be roughly 50/50 at 60 min but much greater than this at short times of incubation of CHO cells. It seems likely that this ratio is much larger than in adipocytes since microscopical data from brown adipose tissue suggest that only 4% of GLUT4 is in the TE pool in the basal state, rising to 12% following insulin treatment. The differences between the pool size of GLUT4 in endosomes of CHO and adipose cells may be due to more efficient removal of

GLUT4 from the sorting early endosomes in 3T3-L1 cells. This may occur because the targeting domains are more efficiently recognized by the sequestration machinery that intracellularly processes the GLUT4. An additional feature of the trafficking behaviour of GLUT4 in insulin-target tissues is of course that it is only in these systems that GLUT4 can be rapidly released from the TV pool in response to the hormone.

We have previously observed that there is only a small difference between the endocytosis rate constants of GLUT4 and GLUT1. Trafficking of these glucose transporters in 3T3-L1 cells showed either no significant difference [35] or only an approx. 50% lower endocytosis rate constant of GLUT1 than of GLUT4 [27,36]. Using a plasma membrane lawn assay, Verhey et al. [24], also report that GLUT4 and GLUT1 leave the cell surface with similar internalization rates. Therefore, data from several studies suggest that the major difference between the trafficking behaviour of GLUT4 and GLUT1 occurs at the intracellular sorting stage leading to a lower constitutive or basal rate of exocytosis of the former isoform. The small difference between the endocytosis rate constants for GLUT4 and GLUT1 in 3T3-L1 cells is consistent with the $\approx 40\%$ slower endocytosis of the N-(YQ-AA) mutant than of wild-type GLUT4 as GLUT1 does not possess the N-terminal targeting information present in GLUT4.

In CHO cells, there is a clear difference between the behaviour of transfected and endogenous GLUT1, the latter behaving in a manner that is consistent with our data on endogenous GLUT1 in 3T3-L1 cells. The transfected GLUT1 is expressed at levels which are ≈ 3 -fold higher than transfected GLUT4, and therefore at this level of expression it may saturate the intracellular sequestration process. Since saturation effects are a potential problem in analysing trafficking behaviour we have studied an additional GLUT4 mutant with lower levels of expression than that of the wild-type clone reported here. The GLUT4 in this low-expression GLUT4 clone behaved in a similar manner to the high-expression GLUT4 clone, suggesting that saturation is less of a potential problem for analysing GLUT4 behaviour. However, we cannot exclude the possibility that the GLUT4 mutants, with similar expression levels to the wild-type GLUT4 clone, exhibit perturbed trafficking due to a saturation phenomenon. Indeed, Marsh et al. [25] suggest that GLUT4 that is substituted in the di-leucine motif only behaves differently from wild-type GLUT4 when the mutant is expressed at high levels in the cell. The intracellular sequestration to the TV pool may have a limited capacity which, when saturated, results in return of transporters to the plasma membrane by default. This effect may account for the high basal glucose transport activity observed when GLUT4 is overexpressed in the adipose tissue of transgenic mice [37].

An additional complexity of interpretation of trafficking behaviour of chimeric and mutated GLUT4 is the extent to which targeting domains may either interfere with, or compensate for, each other at various stages of the trafficking pathway. Therefore, the presence of the N-terminal domain in our C-(LL-AA) mutant may compensate for any reduced recognition of the C-terminal domain at the level of the plasma membrane. On the other hand, it has been argued that the presence of the phenylalanine at the N-terminus of GLUT4 may slow the endocytosis in comparison with the internalization expected for a more avidly internalized β -turn type of motif containing tyrosine. As suggested by Piper et al. [20], a prolonged retention of GLUT4, either in the plasma membrane or in the recycling endosomes, may be beneficial in terms of sustaining an insulin activation of GLUT4 recruitment. Detailed considerations of these potential complexities to the GLUT4 trafficking behaviour cannot be fully

refined from the presently available data but these considerations may provide explanations for phenomena such as the observation of an apparently stronger internalization of a GLUT1/GLUT4 chimera than of wild-type GLUT4 [23].

The targeting behaviour of GLUT4 may be complex but it is clear from data presented here and elsewhere [23–25] that neither the N- nor the C-terminal di-leucine motif alone is entirely sufficient to produce wild-type GLUT4 trafficking behaviour.

This work is supported by the Medical Research Council (U.K.), and by the Monbusyo International Scientific Research Programme.

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