

Distinct Signals in the GLUT4 Glucose Transporter for Internalization and for Targeting to an Insulin-responsive Compartment

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Abstract. In adipose and muscle cells, insulin stimulates a rapid and dramatic increase in glucose uptake, primarily by promoting the redistribution of the GLUT4 glucose transporter from its intracellular storage site to the plasma membrane. In contrast, the more ubiquitously expressed isoform GLUT1 is localized at the cell surface in the basal state, and shows a less dramatic translocation in response to insulin. To identify sequences involved in the differential subcellular localization and hormone-responsiveness of these isoforms, chimeric GLUT1/GLUT4 transporters were stably expressed in mouse 3T3-L1 adipocytes. The NH₂ terminus of GLUT4 contains sequences capable of sequestering the transporter inside the cell, although not in an

insulin-sensitive pool. In contrast, the COOH-terminal 30 amino acids of GLUT4 are sufficient for its correct localization to an intracellular storage pool which translocates to the cell surface in response to insulin. The dileucine motif within this domain, which is required for intracellular sequestration of chimeric transporters in fibroblasts, is not critical for targeting to the hormone-responsive compartment in adipocytes. Analysis of rates of internalization of chimeric transporter after the removal of insulin from cells, as well as the subcellular distribution of transporters in cells unexposed to or treated with insulin, leads to a three-pool model which can account for the data.

GLUCOSE uptake into mammalian cells is accomplished by a family of proteins, the facilitative glucose transporters, which differ in their tissue distribution and physiological roles. Two of the isoforms identified to date, GLUT1 and GLUT4, are expressed in adipose and muscle, those tissues which exhibit a marked increase in glucose uptake in response to insulin (5). The differential localization of these isoforms within the cell may contribute to their distinct functions. GLUT1, which is expressed in virtually all tissues, is distributed to both the plasma membrane and the interior of the cell in the basal state. Insulin causes a two- to fivefold increase in the amount of GLUT1 present at the cell surface, a recruitment similar to other recycling membrane proteins such as the insulin-like growth factor II and transferrin receptors (39). GLUT4, which is expressed exclusively in insulin-responsive cell types, is excluded from the plasma membrane and instead localizes to an intracellular storage pool in the basal state. Insulin stimulates the specific recruitment of these vesicles to the cell surface, increasing by 10- to 40-fold the amount of GLUT4 present at the cell surface, and thereby dramatically increasing the hexose uptake capacity of the cell (8, 9, 21, 24, 34, 36–38, 49).

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The mechanisms responsible for the insulin-regulated movement of GLUT4 in adipose and muscle may be similar to those utilized by other cell types for regulated secretion. This is supported by the ability of GLUT4 to segregate to large dense core vesicles when expressed in the neuroendocrine cell line PC12 (23). The involvement of members of the small G protein family is suggested by the ability of non-hydrolyzable GTP analogs to substitute for insulin in stimulating GLUT4 translocation (3, 31) and by the cloning of an adipocyte-specific homologue of rab3 (rab3D; reference 2). Several other components of the regulated secretion machinery have been identified in adipocytes, including the vesicle associated membrane protein, synaptobrevin (7), and the secretory carrier membrane proteins (26, 40). In addition, the specialized endosome-related subcellular compartment to which GLUT4 is sequestered in adipocytes may be related to the compartment in antigen-presenting cells where major histocompatibility complex class II molecules are loaded with their antigenic peptides (1, 41, 46). Insights into the trafficking of GLUT4 may also be applicable to the study of other regulatable transmembrane transporters. For example, the water channel aquaporin-2 is sequestered in intracellular vesicles and moves to the apical plasma membrane of renal collecting duct cells in response to the antidiuretic hormone vasopressin (13).

The differential localization of GLUT1 and GLUT4 in insulin-responsive tissues has been reproduced in heterol-

ogous cell types into which the cDNAs have been introduced (18, 23, 28, 35). This suggests that the GLUT1 and GLUT4 proteins contain within their primary sequences the information necessary to direct their differential sorting within the cell, and that many cell types are capable of recognizing these sequences. Indeed, the construction of chimeric GLUT1/GLUT4 transporters and their expression in cultured cell lines has led to the identification of sequences involved in differential sorting. Piper et al. have found the NH₂ terminus of GLUT4 necessary for the intracellular sequestration of this isoform in Chinese hamster ovary cells, and a Phe critical, presumably by affecting the internalization of GLUT4 from the cell surface (29, 30). However, several laboratories have concluded that the primary targeting information resides in the COOH-terminal 30 amino acids of the transporters (11, 27, 43). In particular, a dileucine motif within this region is necessary for the intracellular sequestration of GLUT4 in fibroblasts (10, 42). These results suggest that GLUT4, and not GLUT1, contains the actively recognized sorting information, as mutation of the dileucine motif altered the subcellular location of the mutant transporters to one that was indistinguishable from GLUT1.

As informative as the previous studies are, none have investigated the localization of the chimeric transporters in the physiologically relevant cell types, muscle and adipose. In addition, it is not known whether the signals which confer intracellular localization of GLUT4 in the basal state are sufficient to dictate its dramatic translocation to the plasma membrane in response to insulin. To address these questions, we have utilized the mouse cell line 3T3-L1, a derivative of Swiss 3T3 fibroblasts which differentiates into adipocyte-like cells under the appropriate culture conditions. These cells develop many of the characteristics of authentic adipose cells, including marked, acute insulin-responsive glucose transport (32). GLUT1/GLUT4 chimeric transporters were stably expressed in 3T3-L1 adipocytes and the subcellular localization of the transporters was determined in the basal and insulin-stimulated states. Our results suggest that chimeric transporters containing the NH₂ terminus of GLUT4 are localized to the interior of the cell, although not in an insulin-sensitive compartment. In addition, we show that sequences within the COOH-terminal 30 amino acids of GLUT4, but not the dileucine motif, are required for the correct localization of chimeric transporters to an intracellular storage vesicle which is capable of translocating to the cell surface in response to insulin.

Materials and Methods

Materials

Crystalline porcine insulin was a gift from Lilly Research Laboratories (Indianapolis, IN). ¹²⁵I-protein A was purchased from ICN Radiochemicals (Irvine, CA) and 2-[1,2-³H]deoxy-D-glucose (30.6 Ci/mmol) from New England Nuclear/DuPont (Boston, MA). BSA was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

DNA Constructs and Cell Culture

The retroviral expression vector, preparation of virus, and the infection of 3T3-L1 cells have been described previously (23). Construction of the chimeric transporters, the creation of a species-specific epitope tag and the

mutation of Leu489Leu490 were described previously (42). The nucleotide and amino acid numberings are according to the published sequences of rat GLUT1 and GLUT4 (4, 6). 3T3-L1 cells were grown and differentiated as previously described (14). Total membranes were prepared and assayed for expression of chimeric transporter by Western blotting as described previously (23).

Glucose Transport Assays

3T3-L1 fibroblasts were plated in 22-mm wells of 12-well plates, induced to differentiate, and used between days 14–28 after differentiation. Glucose transport, as assayed by the uptake of 2-deoxy-D-[³H]glucose, was measured in duplicate for the last 5 min of a 15-min insulin stimulation (final concentration 100 nM) as described (14). Uptake in the presence of 10 μM cytochalasin-B was subtracted from all values. Lysates were normalized to protein concentration using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL).

Plasma Membrane Sheet Assay

3T3-L1 fibroblasts were plated on glass coverslips, induced to differentiate, and used between days 14–28 after differentiation. Adipocytes were incubated in Leibovitz L-15 medium containing 0.2% bovine serum albumin for 2 h and then stimulated or not with insulin (final concentration 100 nM) for 15 min. Plasma membrane (PM)¹ "sheets" were prepared, fixed and stained for immunofluorescence as described previously (14, 43). For the internalization time course, adipocytes were stimulated with insulin, washed seven times with ice-cold PBS, and then allowed to recover for 0–180 min in Leibovitz L-15 medium containing 1.0% BSA at 37°C before preparation of PM sheets. The sheets were viewed with a Zeiss Axiovert 135M microscope (Carl Zeiss Corp., Thornwood, NY). Images were acquired with a Dage-intensified silicon-intensified-target (ISIT) camera (Dage Electronics, Michigan City, IL) and were captured and analyzed using Image-1/Metamorph software (Universal Imaging Corp., West Chester, PA). For quantitation, the average pixel brightness of at least six fields (10–20 cells/field) was measured for each transporter at each time point. In each field, the average pixel brightness of the slide next to the cells was measured for background levels and subtracted from that of the cells. The measurements at each time point for each transporter were then pooled to obtain means and standard errors.

Mathematical Modeling of Glucose Transporter Trafficking

The theoretical predictions of the time course of glucose transporter trafficking are based on analytical solutions of the rate equations. The rate of movement of transporters out of a compartment equals the product of the rate constant times the amount of transporter in that compartment (first order kinetics) (22). For the three-pool model,

$x_p(t)$ = the fraction of glucose transporters on the PM

$x_{en}(t)$ = the fraction of glucose transporters in endosomes

$x_{irv}(t)$ = the fraction of glucose transporters in IRV

The rate constants k_{endo} , k_1 , k_s , k_4 , and k_2 are indicated in Fig 6. The numerical values for each rate constant are taken from published studies on the trafficking of glucose transporters in 3T3-L1 adipocytes (22, 47–49). Rate constants with an index i represent the insulin-stimulated state. Solution of the differential equations describing the abundance of cell surface glucose transporters, as well as their steady-state distributions with and without hormone, will be described elsewhere (Yeh, J., Verhey, K. J., and Birnbaum, M. J., manuscript submitted for publication).

The half life ($t_{1/2}$) for recovery of subcellular distributions for each transporter after insulin removal is estimated experimentally by first plotting the fraction of maximal response (y) vs. time, and then applying a curve fit of $y = m0e^{m1t}$ such that $t_{1/2} = 0.693/m1$. This is an approximation since the real kinetics are more complicated than a simple exponential function. This estimation is expected to be somewhat smaller than measurements determined for $t_{1/2}$ by a linear interpolation to the point at $y = 0.5$.

1. *Abbreviations used in this paper:* IRV, insulin-responsive vesicles; PM, plasma membrane.

Results

Subcellular Localization of the Chimeric Transporters

As 3T3-L1 adipocytes express endogenous GLUT1 and GLUT4, the analysis of chimeric transporters has been facilitated by the creation of a species-specific epitope "tag" in the central cytoplasmic loop of the transporter (H, Fig. 1) which is recognized specifically by the mAb G3 (43). Conditioned media containing retrovirus encoding chimeric transporters were used to infect 3T3-L1 fibroblasts. At least 50 stable G418-resistant clones of each construct were expanded and screened for expression of chimeric transporter by Western blot analysis of total cellular membranes with mAb G3. In addition, each clone was tested for the ability to differentiate into an adipocyte-like cell, as assayed by the appearance of characteristic lipid droplets by phase contrast microscopy. Two to four clones of each chimeric transporter were selected for further analysis, of which two representative clones are shown in Fig. 2 A. The level of expression of chimeric transporter was quantitated by Western blotting with a polyclonal antibody to the COOH terminus of GLUT4 and was determined to be less than twofold that of the endogenous GLUT4 (Fig. 2 B).

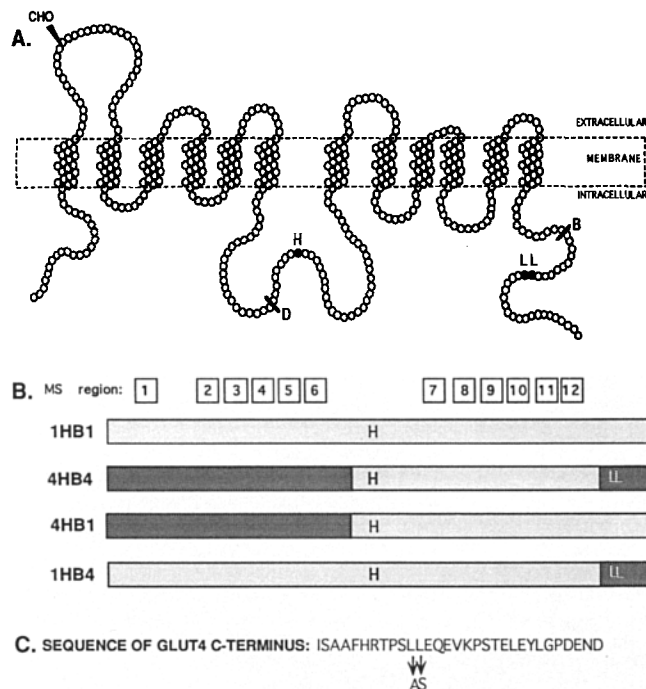
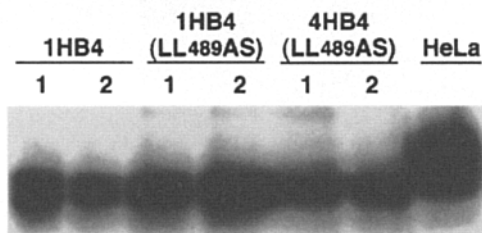
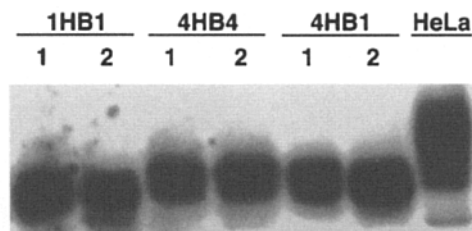


Figure 1. Structures of chimeric transporters. (A) Predicted transmembrane topology of the glucose transporters. The indicated restriction sites HindIII (D) and BglII (B) were utilized to exchange portions of the GLUT1 and GLUT4 cDNAs. H designates the amino acid of GLUT1 altered to create the human epitope tag. LL indicates the LeuLeu residues that were altered to AlaSer in the COOH terminus of GLUT4. (B) Contribution of GLUT1 (light gray bars) and GLUT4 (dark gray bars) sequences to each chimeric transporter. For the naming of the chimeras, the numbers refer to the contribution of GLUT1 or GLUT4 at the NH₂ and COOH termini, H designates the human epitope tag, and B refers to the BglII site. (C) The primary amino acid sequence of GLUT4 following the BglII site. The leucine residues that were mutated are indicated. MS, membrane spanning.

A. mAb G3



B. α -GLUT4

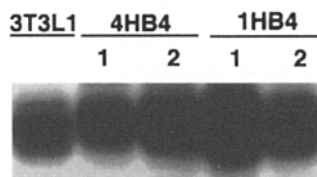


Figure 2. Western blot analysis of clones of 3T3-L1 cells expressing the tagged chimeric transporters. (A) Total membranes from HeLa cells (30 μ g) and from clones of 3T3-L1 fibroblasts stably infected with retrovirus containing the cDNAs encoding the tagged chimeric transporters (40 μ g) were subject to SDS-polyacrylamide gel electrophoresis and Western blotting with mAb G3 followed by ¹²⁵I-protein A. (B) Total membranes from untransfected 3T3-L1 adipocytes and from adipocytes expressing the indicated tagged chimeric transporters (40 μ g) were subject to SDS-polyacrylamide gel electrophoresis and Western blotting with a polyclonal antibody to the COOH terminus of GLUT4 followed by ¹²⁵I-protein A. The reactivity with α -GLUT4 relative to parental 3T3-L1 adipocytes is as follows: 3T3-L1, 1.0; 4HB4 clone 1, 1.15; 4HB4 clone 2, 1.20; 1HB4 clone 1, 1.54; 1HB4 clone 2, 1.33.

The subcellular localization of the chimeric transporters in 3T3-L1 fibroblasts, determined by indirect immunofluorescence microscopy with mAb G3 (Fig. 3), was similar to that in NIH3T3 fibroblasts (42, 43). The transporter 1HB1 displayed the characteristic cell surface staining of GLUT1 in fibroblasts whereas 4HB4 was localized to the perinuclear region of the cell, identical to the localization seen for GLUT4 (23, 43). The chimeric transporter 4HB1, which contains the NH₂-terminal 183 amino acids of GLUT4, showed some predominant staining of the perinuclear region, though there was sometimes slight staining of the periphery of the cell. The chimera 1HB4, which contains the COOH-terminal 30 amino acids of GLUT4, was localized to the perinuclear region of the cell in a pattern indistinguishable from 4HB4 and GLUT4. The mutation of the dileucine motif within the GLUT4 COOH terminus, chi-

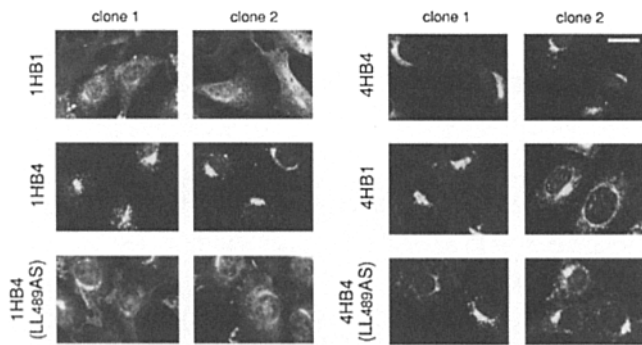


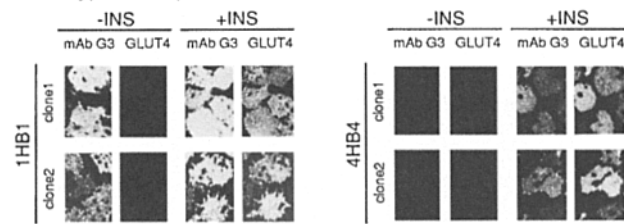
Figure 3. Indirect immunofluorescence of the tagged chimeric transporters in 3T3-L1 fibroblasts. Clones of 3T3-L1 fibroblasts stably expressing the tagged chimeric transporters were fixed, permeabilized, and stained with mAb G3 and rhodamine-conjugated goat anti-mouse secondary antibody. Bar, 20 μ m.

meras 1HB4(LL489AS) and 4HB4(LL489AS), caused a redistribution of the transporter from the perinuclear region to the periphery of the cell, consistent with increased expression on the plasma membrane.

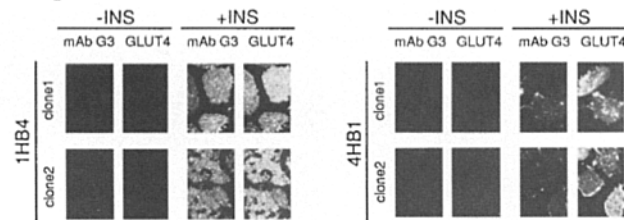
To identify sequences responsible for the subcellular sorting of GLUT4 in its physiologically relevant cell type, the clones expressing chimeric transporters were plated on coverslips, induced to differentiate into adipocytes and the subcellular location of the transporters in the basal and insulin-stimulated states determined by the PM sheet assay (Fig. 4). Since all cell lines express approximately the same level of total heterologous transporter (Fig. 2 A), the absolute amount of chimera on the cell surface also represents its fractional distribution to this compartment. The transporter 1HB1 is present on the plasma membrane in the basal state and increases upon stimulation of the cells by insulin (Fig. 4 A), consistent with the known behavior of endogenous, wild-type GLUT1 (8). Note that immunofluorescence using the anti-GLUT4 antisera in the 1HB1 cell line indicates the distribution of the endogenous GLUT4 with and without exposure of cells to hormone. No staining of the PM sheets by mAb G3 can be seen for 4HB4 in the basal state; however there is intense staining upon stimulation of the cells with insulin similar to the staining pattern of endogenous GLUT4 in the basal and insulin-stimulated states (Fig. 4 A). The chimera 4HB1 shows minimal localization to the PM in the basal state; stimulation of these cells by insulin causes a slight increase in this staining (Fig. 4 B). There is no staining of PM sheets by mAb G3 from cells expressing 1HB4 in the absence of insulin, though stimulation by insulin causes redistribution of this chimeric transporter to the cell surface (Fig. 4 B), similar to that for 4HB4 and GLUT4 (Fig. 4 A). These data suggest that the NH₂ terminus of GLUT4 contains information capable of localizing the transporter to the interior of the cell, although not to a maximally insulin-sensitive compartment. These data also suggest that the COOH terminus of GLUT4 contains information sufficient to target the transporter to an intracellular storage site from which it is recruited to the plasma membrane in response to insulin.

The dileucine motif within the COOH terminus of GLUT4 is necessary for its intracellular sequestration in NIH3T3 and 3T3-L1 fibroblasts (above; reference 42). Upon

A. "wild-type" transporters



B. NH₂- and COOH-terminal chimeric transporters



C. dileucine-based chimeric transporters

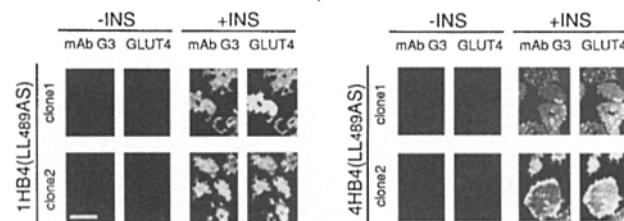


Figure 4. Localization of the tagged chimeric transporters in 3T3-L1 adipocytes in the basal and insulin-stimulated states by labeling of PM sheets. Clones of 3T3-L1 adipocytes stably expressing the tagged chimeric transporters 1HB1 and 4HB4 (A), 1HB4 and 4HB1 (B), and 1HB4(LL489AS) and 4HB4(LL489AS) (C) were stimulated or not with insulin (100 nM, 15 min) and then sonified to prepare PM sheets. These were fixed and labeled with mAb G3 and a rabbit polyclonal antibody to the COOH terminus of GLUT4, and followed by rhodamine-conjugated goat anti-mouse secondary antibody and FITC-conjugated goat anti-rabbit secondary antibody, respectively. Bar, 20 μ m.

differentiation of these cells to adipocytes, however, the mutant transporters 1HB4(LL489AS) and 4HB4(LL489AS) no longer were detected at the PM by mAb G3 in the basal state (Fig. 4 C). Not only are the mutant transporters absent from the cell surface, but they are sequestered inside the cell in a storage compartment capable of insulin-stimulated translocation to the plasma membrane to a level comparable to 4HB4 (Fig. 4 C).

Adipocytes Expressing the Chimeric Transporters Accumulate Hexose at Increased Rates

Two criteria have been used historically to demonstrate the validity of 3T3-L1 adipocytes as an authentic insulin-responsive cell line: insulin-stimulated translocation of GLUT4 to the cell surface (shown in these studies by the PM sheet assay) and a rapid increase in the rate of hexose uptake (8, 32). Subcloning of 3T3-L1 cells often results in subpopulations which have quite variable rates of hexose uptake, particularly after exposure to insulin (17). In this study, selection of G418-resistant clones on the basis of

their capacity to differentiate efficiently tended to improve the ability of insulin to activate hexose uptake (Table I). However, despite clonal differences, the data shown in Table I are consistent with the PM sheet assay. Insulin stimulated hexose uptake almost 10-fold in untransfected 3T3-L1 adipocytes. Adipocytes expressing the chimeric transporter 1HB1 displayed elevated levels of both basal and insulin-stimulated hexose uptake compared to parental cells, as reported previously for GLUT1 (19). This resulted in a lower-fold stimulation and presumably reflects the cell surface localization of this transporter in the basal state. Expression of the chimeric transporter 4HB1 resulted in basal and insulin-stimulated levels of hexose uptake similar to those of untransfected adipocytes. This is consistent with both the primarily intracellular localization of this transporter in the basal state and its minimal insulin-stimulated translocation to the cell surface. Adipocytes expressing the chimeric transporters 4HB4, 1HB4, or 1HB4(LL489AS) showed levels of basal transport comparable to parental cells; however, a larger degree of insulin-stimulated transport than in the untransfected adipocytes was observed, resulting in a larger fold stimulation. This most likely corresponds to the increased level of "GLUT4" in these cells as seen by staining PM sheets of cells expressing these chimeras in the insulin-stimulated state.

The Effect of the Dileucine Motif in the COOH Terminus of GLUT4

A dileucine motif has been suggested as comprising a critical part of the sorting signals of several membrane proteins, and has been implicated in sorting from the TGN, the PM and endosomes (for review see reference 33). Since mutation of the dileucine motif in the COOH terminus of GLUT4 did not affect its correct localization to an insulin-sensitive storage compartment in adipocytes (above), we sought to determine whether this motif was instead affecting the sorting of the transporters at the PM after translocating in response to insulin. Adipocytes expressing the chimeric transporters were stimulated, washed extensively at 4°C to remove insulin, and allowed to recover at 37°C for the times indicated in Fig. 5 before PM sheets were prepared. PM sheets of untransfected 3T3-L1 adipocytes showed staining for the endogenous GLUT1 in the basal state, increased staining in response to insulin, and a rapid re-equilibration to basal levels upon insulin removal (Fig. 5 A). Virtually identical results were obtained when staining for the chimeric transporter 1HB1 with mAb G3

Table I. 2-deoxy-Glucose Uptake (pmol/mg/min)

Cell line	-Insulin	+Insulin	Fold stimulation
3T3L1	42.1 (± 3.0)	329.1 (± 10.5)	7.8
1HB1	108.7 (± 24.9)	379.7 (± 21.9)	3.5
4HB4	27.4 (± 4.9)	442.1 (± 69.7)	16.2
4HB1	59.6 (± 15.4)	421.9 (± 20.8)	7.1
1HB4	30.5 (± 4.3)	597.7 (± 56.1)	19.6
1HB4(LL489AS)	38.7 (± 5.3)	482.9 (± 5.8)	12.5

Untransfected 3T3-L1 adipocytes and clones of 3T3-L1 adipocytes stably expressing the tagged chimeras were stimulated or not with insulin (100 nM, 15 min) and hexose uptake was measured in duplicate for the last 5 min. Hexose uptake for each clone was determined in one to four experiments, and the data from the clones of each chimera were pooled (three to six total samples/chimera). Values are expressed as the mean (± SEM).

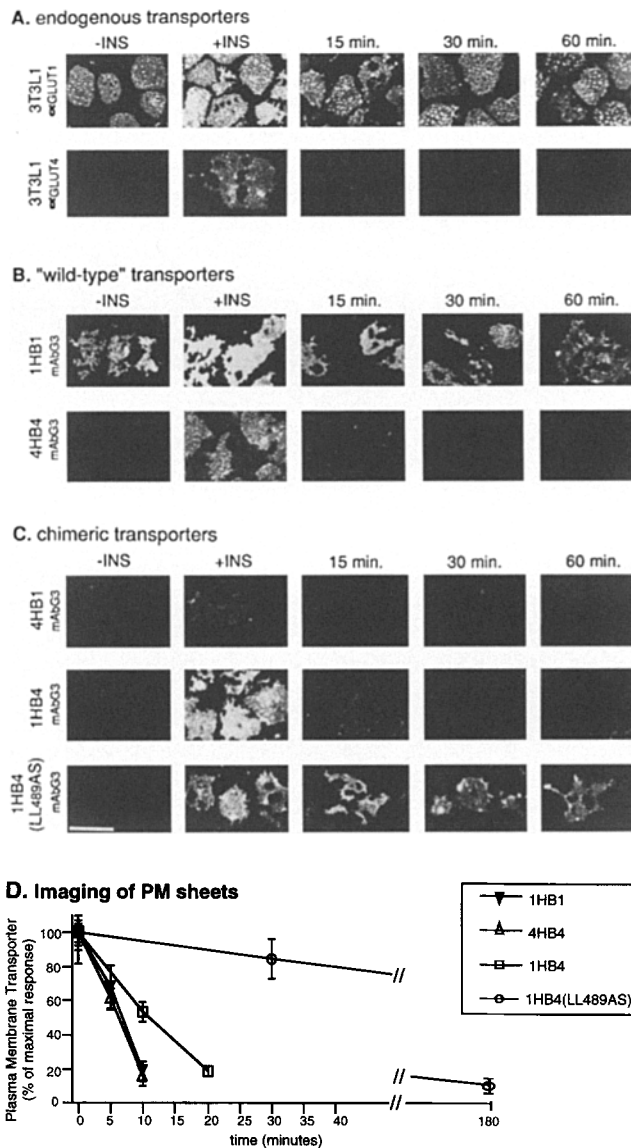


Figure 5. Time course of the cell surface localization of the tagged chimeric transporters after the removal of insulin by labeling of PM sheets. Untransfected 3T3-L1 adipocytes (A) and clones of 3T3-L1 adipocytes stably expressing the tagged chimeras 1HB1 and 4HB4 (B), or 4HB1, 1HB4, and 1HB4(LL489AS) (C) were stimulated or not with insulin (100 nM, 15 min). The cover slips were washed with ice-cold PBS to remove insulin and then returned to 37°C for 0, 15, 30, or 60 min before PM sheets were prepared and stained with a polyclonal antibody to GLUT1 or GLUT4 (A) or with mAb G3 (B and C) followed by rhodamine-conjugated secondary antibodies. (D) Fluorescent staining of PM sheets was quantitated using Image1/Metamorph image processing software. Data are presented as the percent of change in PM staining due to insulin for each chimera, and represent the mean (± SEM) of six fields (10–20 cells/field). Bar, 20 μm.

(Fig. 5 B). PM sheets of untransfected adipocytes showed no staining for endogenous GLUT4 in the basal state, a large increase upon insulin stimulation, and a rapid decrease after the removal of insulin, such that the GLUT4 staining returned to basal levels within 30 min (Fig. 5 A). This corresponds to the rapid decrease in cell surface GLUT4 and glucose uptake after removal of insulin (34,

47). Virtually identical results were found for the chimeric transporters 4HB4 and 1HB4 as ascertained with mAb G3 (Fig. 5, B and C, respectively). The relatively small amount of transporter 4HB1 which redistributed to the cell surface, though difficult to measure precisely, appeared to be rapidly removed upon washing away insulin (Fig. 5 C). The mutant transporter 1HB4(LL489AS) showed staining patterns similar to those of 1HB4 in the basal and insulin-stimulated states; however, the abundance of 1HB4 (LL489AS) on the PM sheets decreased quite slowly after the removal of insulin (Fig. 5 C). The transporter 1HB4 (LL489AS) was undetectable on PM sheets 3 h after the removal of insulin (Fig. 5 D; and data not shown).

Prediction of Glucose Transporter Distributions by Computer Simulation

Computer simulation of GLUT4 trafficking using the rate constants shown in Fig. 6 predicts that GLUT4 changes in abundance at the cell surface from 0.9 to 41.1% after insulin stimulation and is internalized with a $t_{1/2} = 5$ min after insulin removal (Fig. 6). These values are similar to the reported data for GLUT4 trafficking in 3T3-L1 adipocytes and are consistent with the distribution of GLUT4 in brown adipose tissue as visualized by electron microscopy (36, 37, 47, 49). 4HB4 displays a significant movement to the cell surface after exposure of cells to insulin, and a measured $t_{1/2}$ of reinternalization following hormone withdrawal of 3.8 min (Figs. 5 and 6). A similar analysis for GLUT1 and 1HB1 indicates that the behavior of heterologous, epitope-tagged chimeric transporters is indistinguishable from that reported for the corresponding endogenous wild-type carriers and closely matches the predicted values (compare Figs. 4 and 5 to the predicted values in Fig. 6).

Fig. 6, B and E show the predicted $t_{1/2}$ for steady-state subcellular distributions and reinternalization, respectively, for the chimeric transporters based on assignment of the information encoding targeting to the insulin-responsive vesicles (IRV) (k_s) exclusively to the COOH terminus of GLUT4. As can be seen, the experimental data match the calculated values within the limits of measurement. The small amount of 4HB1 at the PM under basal conditions predicted by the model is barely detectable by the sheet assay, and therefore the $t_{1/2}$ after insulin removal is difficult to measure. The effect of LeuLeu residues in the COOH terminus of GLUT4, shown to be important for the correct intracellular sequestration of the transporter in fibroblasts (10, 42), is shown in Fig. 7. Dileucine motifs have been implicated in sorting of membrane proteins from the PM to endosomes as well as from the *trans*-Golgi network to endosomes (33). Fig. 7 A shows the predicted distributions of the transporters if mutation of LeuLeu were to affect their trafficking between the PM and endosomes, but not change k_s or k_4 . We have performed computer simulation of mutant transporter trafficking assuming that alteration of the dileucine motif affects the distribution of transporter by either decreasing k_{endo} or by increasing k_1 (Fig. 7). Either mechanism predicts retention of the mutant transporter in the basal state and significant translocation in response to insulin, as we have found experimentally (Fig. 4 C). However, modeling an effect of

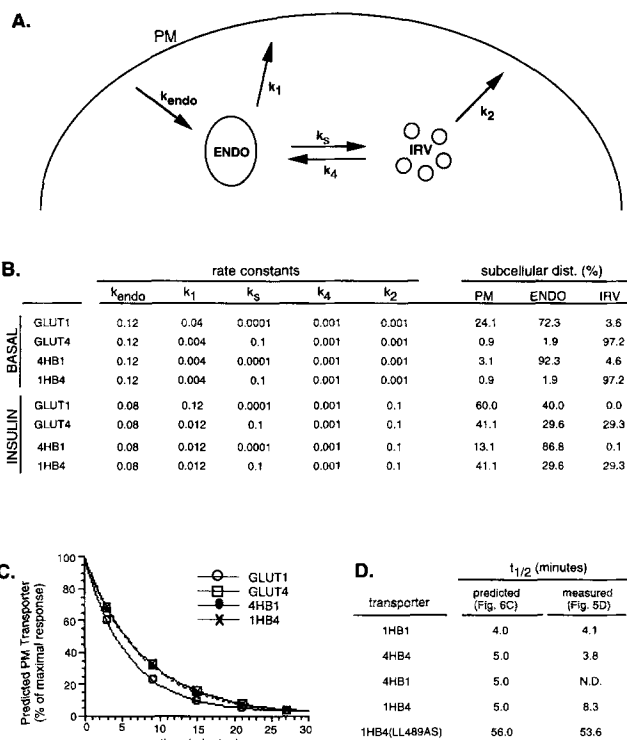


Figure 6. Model for the subcellular trafficking of glucose transporters in adipocytes. (A) Three-pool model for transporter trafficking. k_{endo} , rate of endocytosis. k_1 , rate of recycling to the cell surface. k_s , rate of sequestration in IRV. k_4 , rate of recycling to endosomes. ENDO, endosomes. IRV, insulin-responsive vesicles. (B) Assigned rate constants for transporter trafficking and predicted subcellular distributions of chimeric transporters in the basal and insulin-stimulated states. (C) Computer simulation of transporters remaining at the cell surface after the removal of insulin. (D) *pred.*, each predicted $t_{1/2}$ was calculated as described in the Methods using the constants. *meas.*, the measured $t_{1/2}$ were determined from the data in D. N.D., not determined. The level of 4HB1 present in the PM sheets in insulin-stimulated state is too low to permit calculation of kinetic parameters for the trafficking of this chimeric transporter.

the LL489AS mutation on k_{endo} predicts a marked inhibition in the rate of internalization of chimera from the cell surface following withdrawal of insulin, consistent with the experimental data (Fig. 6 D).

Discussion

The expression of GLUT1 and GLUT4 in several cell types has demonstrated that, in spite of 65% identity in amino acid sequence, each isoform possesses distinct information conferring subcellular targeting (18, 23, 28, 35). Experimental strategies based on the construction of chimeric transporter proteins have led to identification of several structural domains implicated as critical to the intracellular sequestration of GLUT4 (11, 27, 29, 30, 43). However, a major limitation of the previous studies has been that none of the cells into which the transporters have been introduced represents a physiologically meaningful model system as defined by two criteria: expression of significant quantities of the endogenous GLUT4 glucose transporter and an insulin-stimulatable augmentation

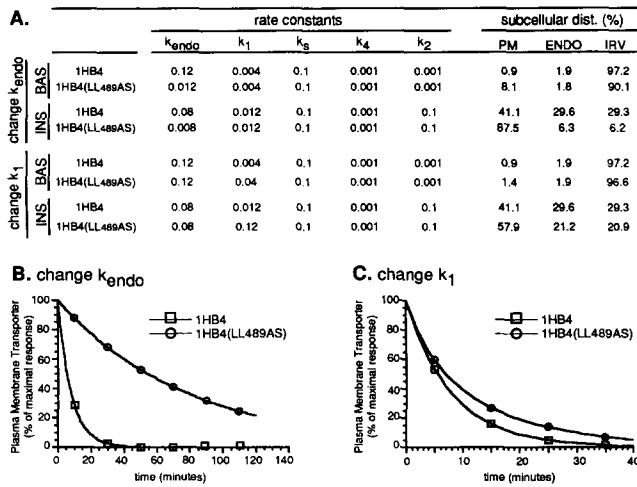


Figure 7. Model for the subcellular trafficking of 1HB4 and 1HB4(LL489AS) in adipocytes. (A) Assigned rate constants and resultant subcellular distributions of the transporters in the basal and inulin-stimulated states if the dileucine mutation affects either k_{endo} or k_1 . BAS, basal. INS, insulin. (B) Computer simulation of transporters remaining at the cell surface after insulin removal if the dileucine mutation affects k_{endo} . (C) Computer simulation of transporters remaining at the cell surface after insulin removal if the dileucine mutation affects k_1 .

in hexose uptake comparable to the *in vivo* target tissue. Numerous published studies have affirmed the validity of the 3T3-L1 adipocyte as a model for hormone-responsive fat cells (8, 15, 32). The development of the differentiated phenotype may well be accompanied by the accumulation of adipocyte-specific proteins which specifically associate with GLUT4 and participate in either its sorting to an hormone-regulatable intracellular storage vesicle or insulin-dependent translocation to the plasma membrane. Consistent with these ideas, we have found our own data on the steady-state distributions of recombinant transporters in NIH-3T3 fibroblasts to be either predictive of the behavior of chimeras in adipocytes, or misleading, depending upon the signal. Thus, the present study has confirmed that the COOH-terminal 30 amino acids of GLUT4, identified as important for subcellular sorting by expression of chimeric transporters in undifferentiated cell types (11, 27, 43), are also critical for trafficking in adipocytes. However, the dileucine motif contained within this region of the transporter which is crucial for intracellular sequestration in fibroblasts (10, 42), was not required for hormone responsiveness in adipocytes, although it may contribute to the rate at which GLUT4 returns to the cell interior after withdrawal of insulin.

A major obstacle to determining the basal subcellular localizations of GLUT1 and GLUT4, as well as their redistributions in response to insulin, has been the difficulty in obtaining pure membrane fractions from cultured cells. Classical cell fractionation seriously underestimates the recruitment of GLUT4 to the plasma membrane, almost certainly due to contamination of the PM with intracellular membranes (8, 19, 45). The use of immunoelectron microscopy, while allowing the demonstration of marked insulin-dependent GLUT4 translocation, has also been associated with misleading results due to non-specific label-

ing and epitope-masking anomalies (37, 38, 44). The use of the cell-impermeant photolabel ATB-BMPA (2-*N*-4-[1-azido-2,2,2-trifluoroethyl]benzoyl-1,3-bis(*D*-mannos-4-yl)oxy)-2-propylamine) has contributed significant information about trafficking of transporters, but has been criticized for potential artifacts related to its binding only to catalytically active transporters (12, 22). In these studies, we have elected to utilize the PM sheet assay to directly ascertain the abundance of transporters at the cell surface (14, 20, 30, 31, 43). Since cell lines were selected for relatively equivalent levels of expression of heterologous transporter, measurement of surface transporter allows extrapolation of the fractional distribution of a given chimera between the plasma and intracellular membranes. Robinson et al. (31) have clearly demonstrated that PM sheets are essentially devoid of intracellular organelles, and we have found that the abundance of GLUT4 on the attached surface of the adipocyte reflects an equivalent density of transporter on the apical plasma membrane (Hausdorff, S. F., and M. J. Birnbaum, unpublished observations). Quantitation of the PM sheet assay leads to a magnitude of insulin-stimulated GLUT4 translocation comparable to that found by immunoelectron microscopy in white adipose tissue and ATB-BMPA labeling in 3T3-L1 adipocytes (8, 31, 38). Thus we believe that the subcellular localizations of GLUT1 and GLUT4 as assessed by the PM sheet assay are an accurate reflection of the processes occurring in the intact adipocyte, with the reservation that it is difficult to quantitate low levels of transporter on the cell surface. The most compelling argument in support of the validity of this assay is the congruence between our data and that obtained with other techniques studying endogenous, wild-type glucose transporters (see below).

As has been postulated in the past, we favor a model in which GLUT1 and GLUT4 are distributed among three compartments in adipocytes: the plasma membrane, a non-specialized endosomal compartment, and insulin-responsive vesicles (Fig. 6 A) (5, 22). In the basal state, there is an isoform-specific bias in the distribution of transporter between endosomes and the two other communicating compartments. GLUT4 has a greater tendency than GLUT1 to remain in endosomes as opposed to the cell surface. Based on the data presented in this communication, we suggest that this propensity is conferred by information in both the NH₂ and COOH termini of the GLUT4 protein. In addition, GLUT1 is virtually excluded from IRV whereas GLUT4 is rapidly and efficiently sorted to this storage pool. The signal dictating targeting to IRV is encoded exclusively by a dileucine-independent signal in the COOH-terminal 30 amino acids.

Chimeric transporters fusing the NH₂ terminus of GLUT4 to the COOH-terminus of GLUT1 have been reported to localize to the interior of CHO fibroblasts (30). A phenylalanine residue within this region has been suggested to function in the internalization of transporters from the cell surface (16, 29). The chimeric transporter 4HB1, which contains the NH₂-terminal 183 amino acids of GLUT4, also showed some intracellular localization in NIH3T3 and 3T3-L1 fibroblasts (43; and Fig. 3), though other investigators have found this chimeric transporter located primarily at the cell surface in COS cells and *Xenopus* oocytes (11, 27). In 3T3-L1 adipocytes, the chimeric transporter 4HB1

was detected primarily in intracellular compartments in the basal state, and only slightly redistributed to the cell surface in response to insulin (Figs. 4 and 5). We interpret this as indicating that the amino terminus contributes sufficient information to allow removal of transporters from the cell surface, but not sorting to the insulin-responsive compartment. The simplest explanation is that 4HB1 resides in endosomes in the basal state and undergoes a modest translocation in response to insulin.

As in several studies involving fibroblasts, the COOH-terminal 30 amino acids of GLUT4 was sufficient to dictate intracellular sequestration of chimeric transporters in 3T3-L1 fibroblasts and adipocytes (11, 27, 43) (Fig. 4). In the fat cell, 1HB4 shows insulin-stimulatable recruitment to the cell surface comparable to both wild-type endogenous GLUT4 and 4HB4 (Figs. 4 and 5), suggesting that the COOH terminus of GLUT4 contains the necessary information to target the transporter to the IRV. The LeuLeu residues within this region have been shown to be important for efficient intracellular sequestration of the transporter in fibroblasts (10, 42). In adipocytes, the mutant transporters 4HB4(LL489AS) and 1HB4(LL489AS) are intracellular in the basal state and move to the cell surface upon stimulation of the cells by insulin (Fig. 4); we consider this evidence of their localization in IRV in the basal state. After removal of insulin, these mutants are reinternalized slower than the comparable chimera with an intact dileucine, indicating that this motif does have some role in sorting in adipocytes (Fig. 5). A plausible explanation is that the LeuLeu-based motif has a role analogous to the NH₂-terminal signal in favoring distribution of GLUT4 in endosomes as opposed to the plasma membrane. In any case, these data clearly show that sequences in the COOH terminus of GLUT4 distinct from the dileucine motif are involved in sorting to the IRV.

To test our model of glucose transporter trafficking and the sorting signals we postulate, we referred to the extensive mathematical analysis by Holman and coworkers based on their studies with the membrane-impermeant photoaffinity reagent ATB-BMPA (22). Our results with chimeric transporters support their conclusion excluding a single intracellular pool of transporters; instead, there must be at least two pools of transporters inside the cell, distinguishable by their capacities to respond to insulin by translocating to the cell surface. Our model, which is shown in Fig. 6, was developed with the following considerations. First, we have assigned rate constants for wild-type transporter trafficking based on experiments performed in 3T3-L1 adipocytes (47, 49). Second, Holman et al. (22), who only considered the trafficking of GLUT4, assumed a non-reversible movement of transporter among the three pools and set the rate constants k_1 and k_4 equal to 0; however, since GLUT1 is likely to move from endosomes directly to the cell surface, we set $k_1 = 0.04$. Third, since several studies have indicated that GLUT1 and GLUT4 have identical rates of endocytosis, $k_{\text{endo}} = 0.12$ for all transporters in the basal state (47, 49). Insulin has been reported to decrease k_{endo} , so we set $k_{\text{endo}} = 0.08$ in the insulin-stimulated state, similar to the 30% decrease measured in 3T3-L1 adipocytes (49). Fourth, insulin stimulation affects the translocation of proteins from both intracellular pools to the cell surface, although not equally.

Thus, the rate of translocation from endosomes (k_1) increases threefold upon insulin stimulation, consistent with the behavior of GLUT1 in adipocytes and GLUT4 in fibroblasts, whereas the rate of translocation from insulin-responsive vesicles (k_2) increases 100-fold (22, 25).

All rates of chimeric and mutant transporter trafficking we have measured in these experiments are consistent with a three-pool model with targeting dictated by the following sorting signals: (a) a decreased cycling of GLUT4 compared to GLUT1 from endosomes to the plasma membrane, due to information residing in the two cytoplasmic ends of the former transporter, (b) a dileucine-independent signal in the COOH terminus of GLUT4 governing targeting to IRV; and (c) the effect of ablation of the dileucine leading to a decrease in the rate of endocytosis of the mutant transporter. Thus, the virtual exclusion of GLUT4 from the cell surface in the basal state is due to two factors: retention in endosomes and specific sorting to IRV. In the non-insulin-responsive cells investigated previously, it is likely that only the first mechanism is operative, that is, the IRV and sorting of proteins to it are adipocyte specific.

One final caveat should be stated. We have referred to IRV as a specialized compartment, the implication being the existence of a distinct organelle. However, a mechanism by which GLUT4 is specifically sequestered within the endosome, and then escorted to the cell surface in response to insulin, would be consistent with the data and hypotheses presented in this report.

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